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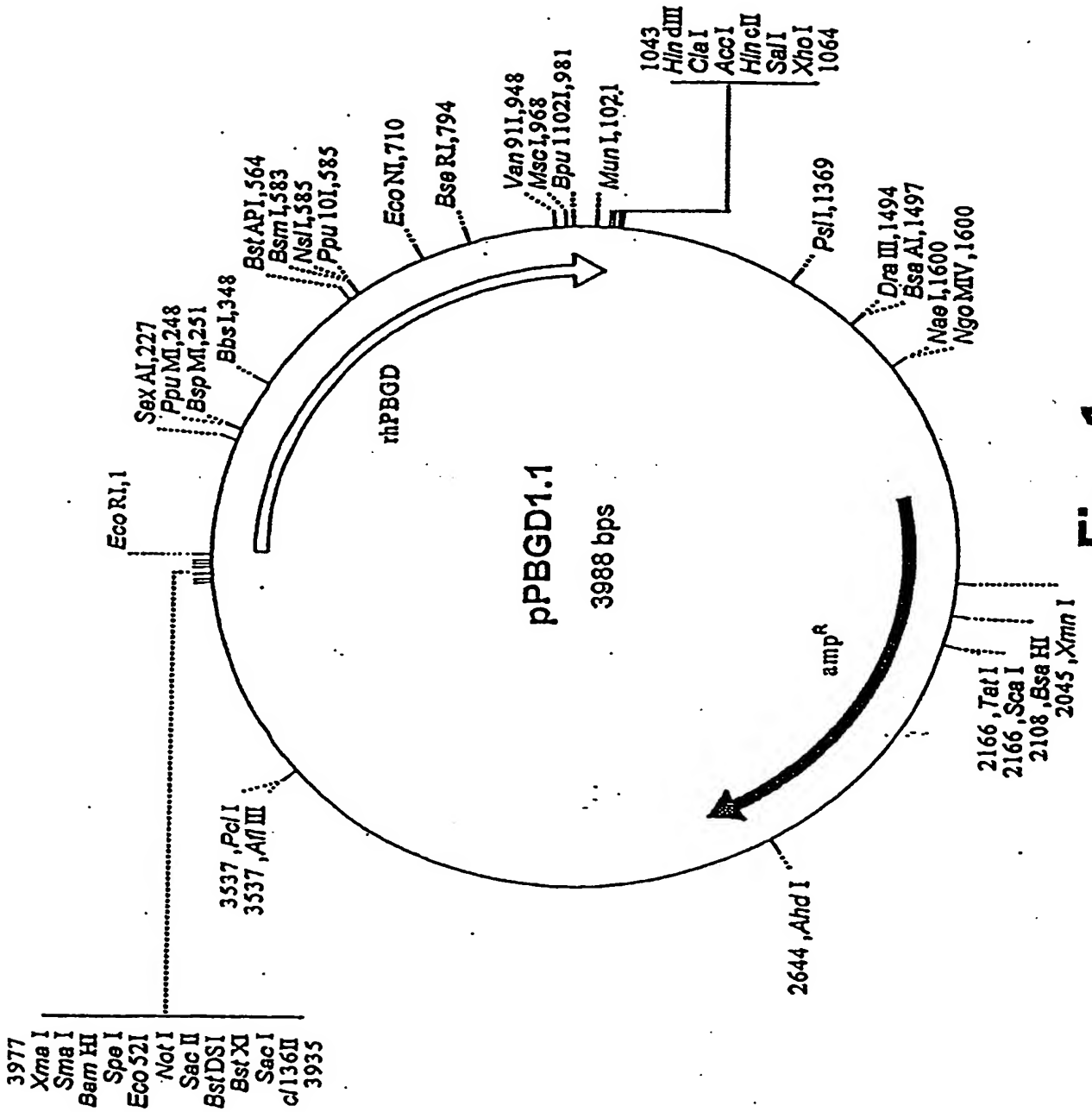

pPBGD1.1
3988 bps
rhPBGD
amp^R
1043 Hin dIII
Cla I
Acc I
Hin cII
Sal I
Xho I
1064
Van 911,948
Msc 1,968
Bpu 11021,981
Mun I,1021
Eco NI,710
Bse RI,794
Bst API,564
Bsm I,583
Nsi I,585
Ppu 10I,585
Sex AI,227
Ppu MI,248
Bsp MI,251
Bbs I,348
Eco RI,1
Psi I,1369
Dra III,1494
Bsa AI,1497
Nae I,1600
Ngo MIV,1600
2166 ,Tat I
2166 ,Sca I
2108 ,Bsa HI
2045 ,Xmn I
2644 ,Ahd I
3537 ,Pcl I
3537 ,Afl III
3977
Xma I
Sma I
Bam HI
Spe I
Eco 52I
Not I
Sac II
Bst DSI
Bst XI
Sac I
cl136II
3935

Fig. 1

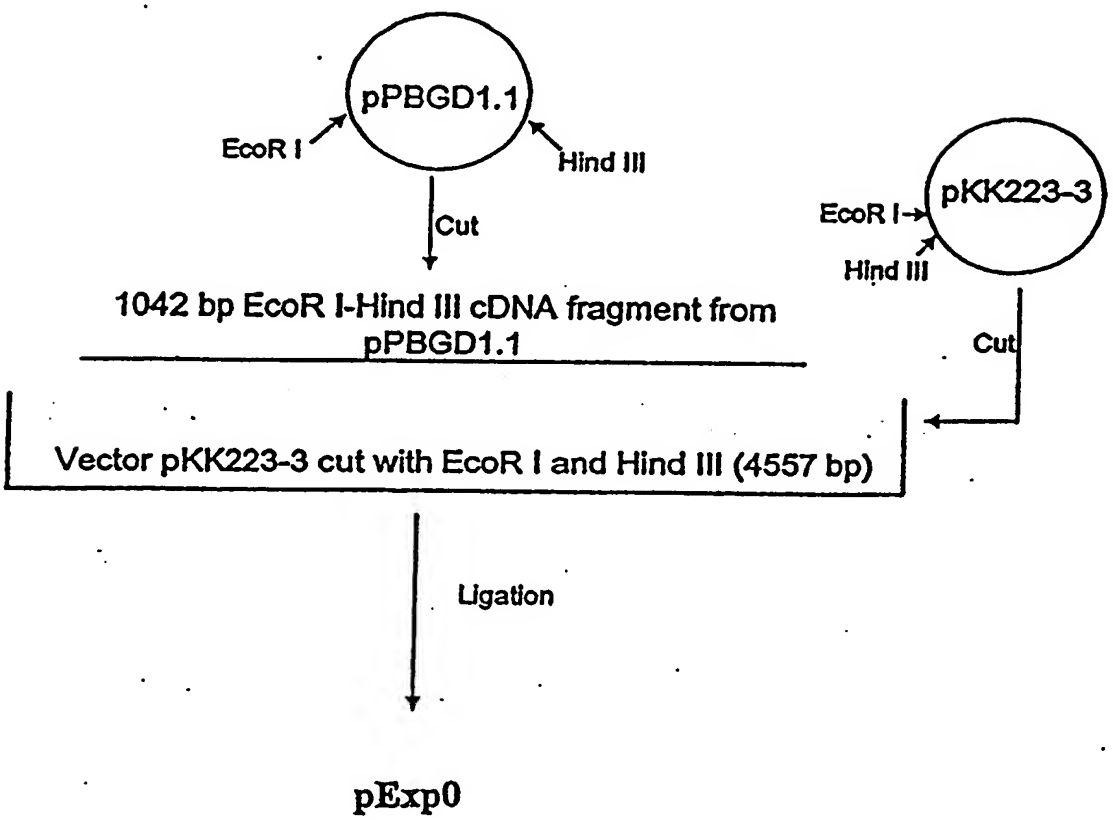

pPBGD1.1
EcoR I
Hind III
Cut
1042 bp EcoR I-Hind III cDNA fragment from pPBGD1.1
pKK223-3
EcoR I
Hind III
Cut
Vector pKK223-3 cut with EcoR I and Hind III (4557 bp)
Ligation
pExp0

Fig. 2

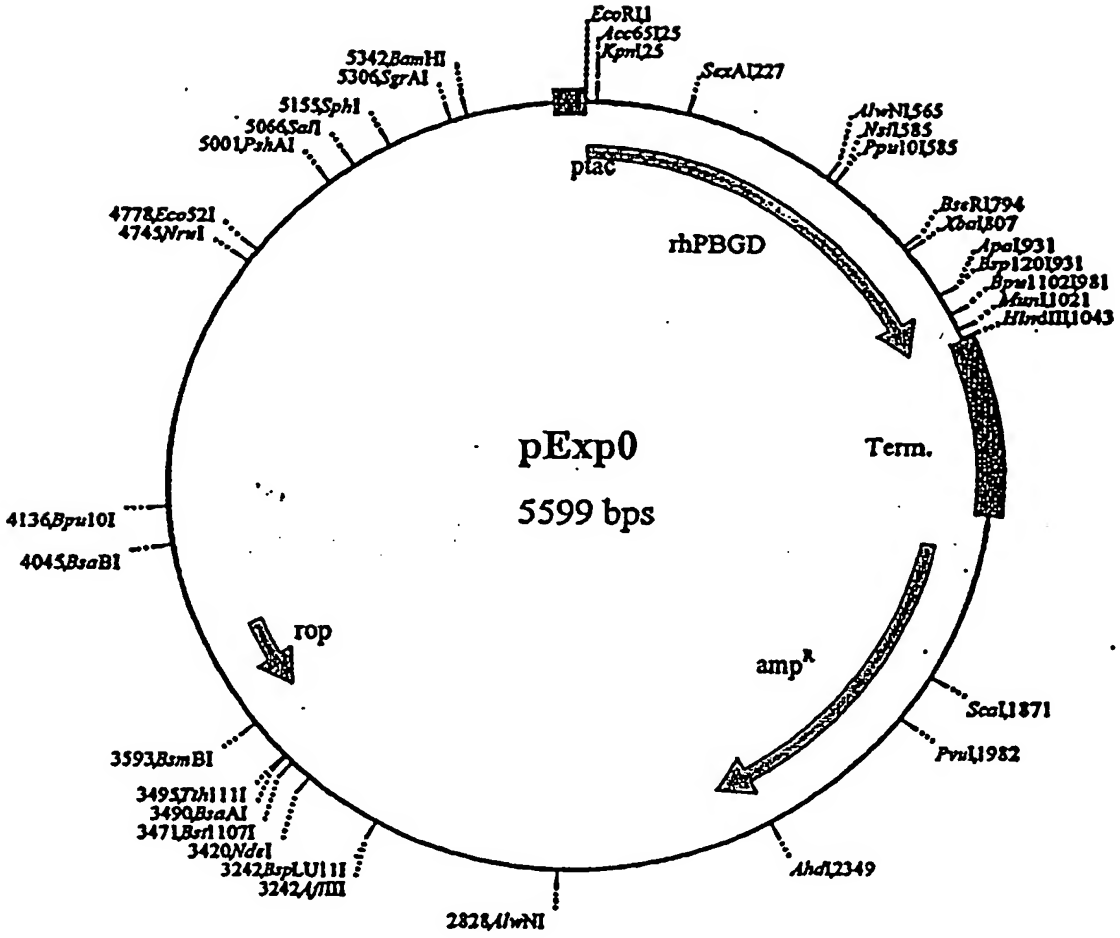

EcoRI1
Acc65I25
KpnI25
ScrAI227
AlwNI565
NsiI585
Ppu10I585
BsrBRI794
XbaI807
ApaI931
Bsp120I931
Bpu1102I981
MunI1021
HindIII1043
ptac
rhPBGD
pExp0
5599 bps
Term.
amp^R
ScaI1871
PvuI1982
AhdI2349
2828AlwNI
3242AflIII
3242BspLU11I
3420NdeI
3471BsrI1071
3490BsaAI
3495NhiIIII
3593BsmBI
rop
4045BsaBI
4136Bpu10I
4743NruI
4778Eco52I
5001PshAI
5066SalI
5155SphI
5306SgrAI
5342BamHI

Fig. 3

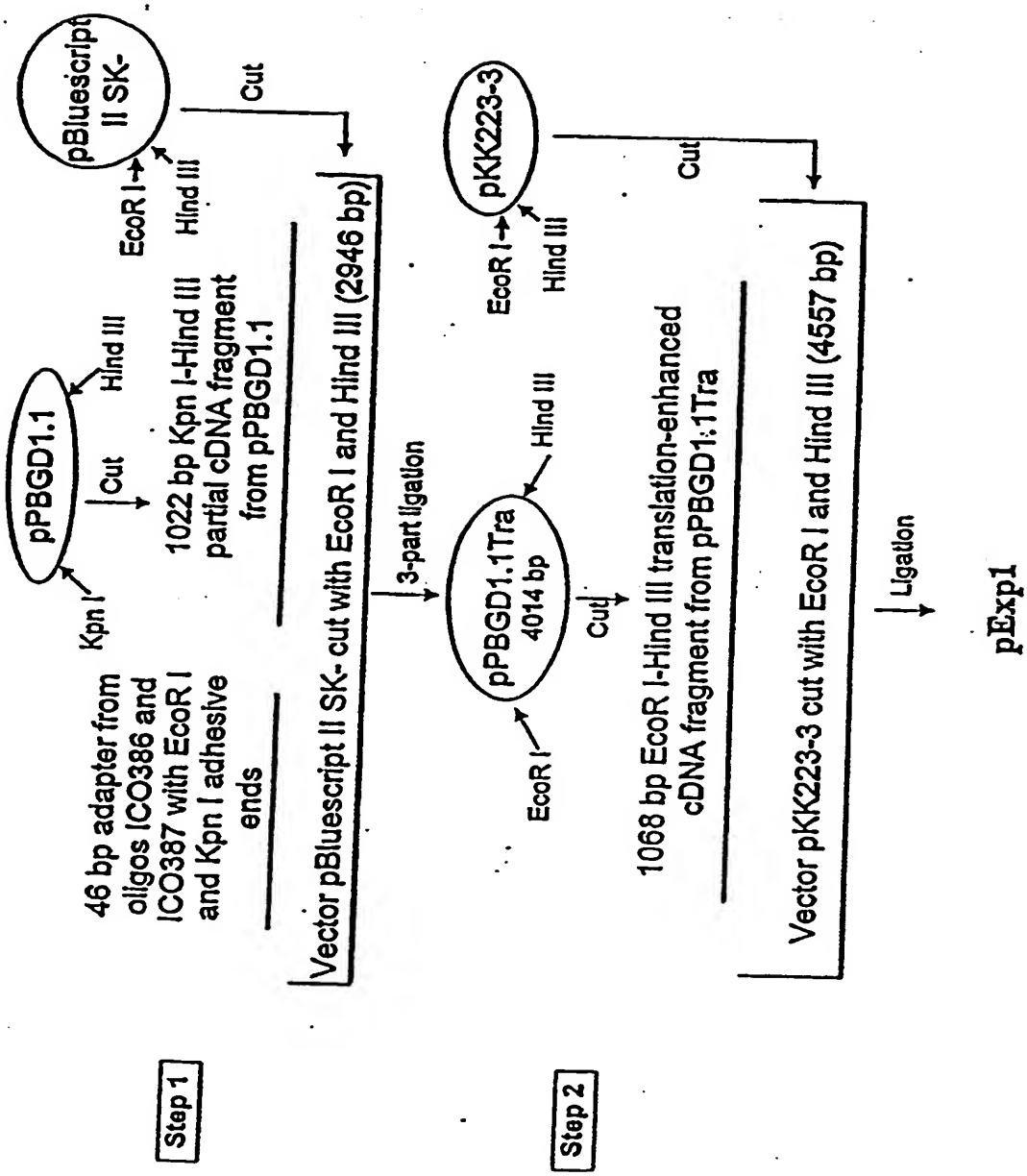

pBluescript II SK-
Cut
EcoR I
Hind III
pPBGD1.1
Hind III
Cut
Kpn I
46 bp adapter from oligos ICO386 and ICO387 with EcoR I and Kpn I adhesive ends
1022 bp Kpn I-Hind III partial cDNA fragment from pPBGD1.1
Vector pBluescript II SK- cut with EcoR I and Hind III (2946 bp)
3-part ligation
Step 1
pPBGD1.1Tra 4014 bp
Hind III
EcoR I
Cut
pKK223-3
Cut
EcoR I
Hind III
1068 bp EcoR I-Hind III translation-enhanced cDNA fragment from pPBGD1:1Tra
Vector pKK223-3 cut with EcoR I and Hind III (4557 bp)
Ligation
pExp1
Step 2

Fig. 4

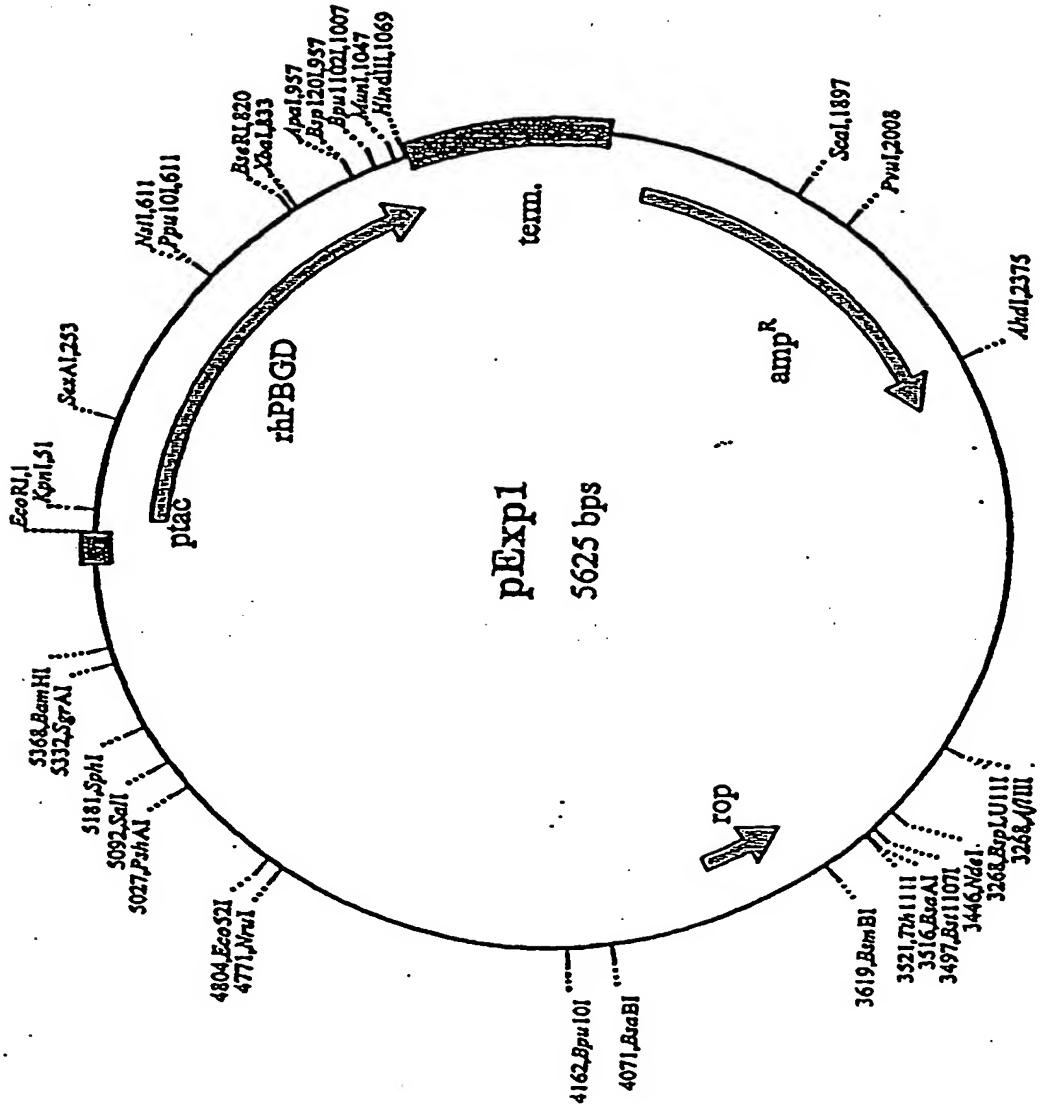

pExp1
5625 bps
rhPBGD
ptac
term.
amp^R
rop
EcoRI,1
KpnI,51
SexAI,233
NsiI,611
Ppu101,611
BseRI,820
XbaI,833
ApaI,957
Bsp1201,957
Bpu11022,1007
XmnI,1047
HindIII,1069
ScaI,1897
PvuI,2008
AhdI,2375
BspLU11I,3268
AflIII,3268
BsmBI,3619
Tth111I,3521
BsaAI,3516
Bst11071,3497
NdeI,3446
4162,Bpu101
4071,BsaBI
4804,Eco521
4771,NruI
5027,PshAI
5092,SalI
5181,SphI
5332,SgrAI
5368,BamHI

Fig. 5

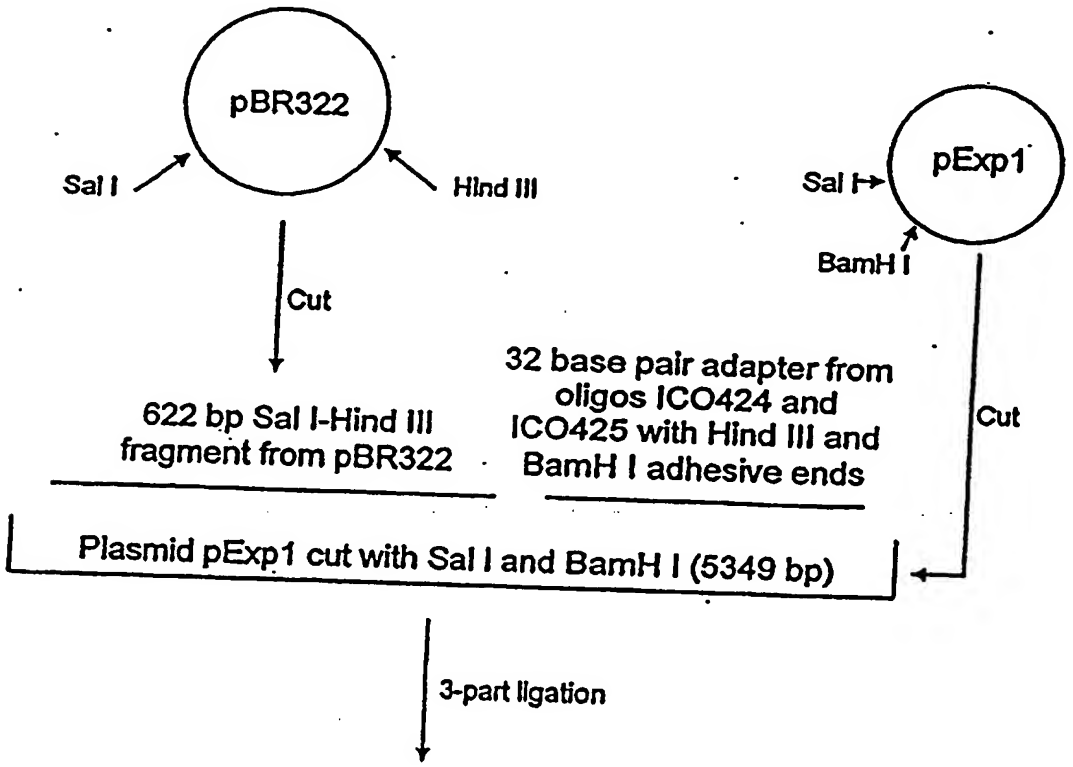

pBR322
Sal I
Hind III
Cut
622 bp Sal I-Hind III fragment from pBR322
pExp1
Sal I
BamH I
32 base pair adapter from oligos ICO424 and ICO425 with Hind III and BamH I adhesive ends
Cut
Plasmid pExp1 cut with Sal I and BamH I (5349 bp)
3-part ligation
pExp1-M2

Fig. 6

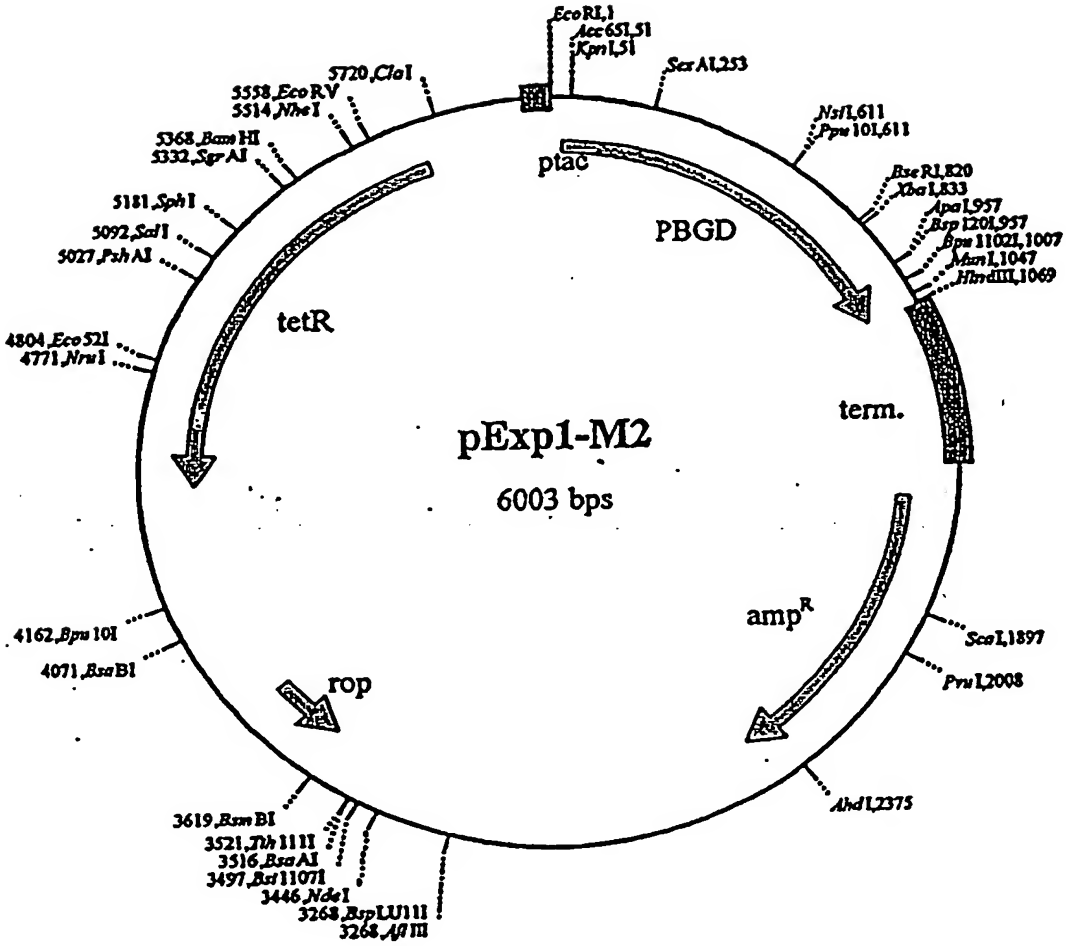
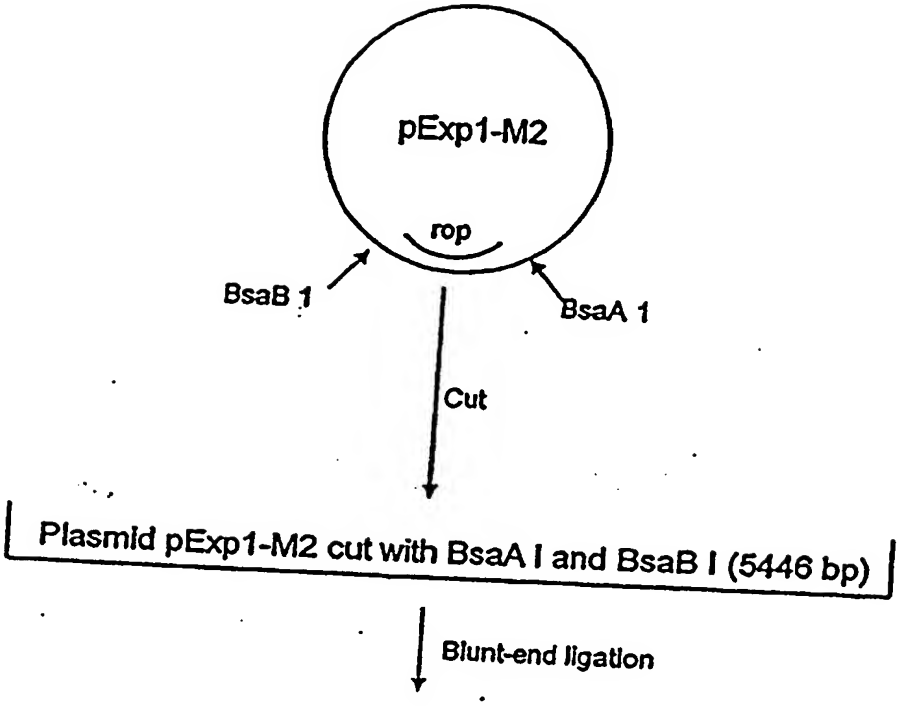
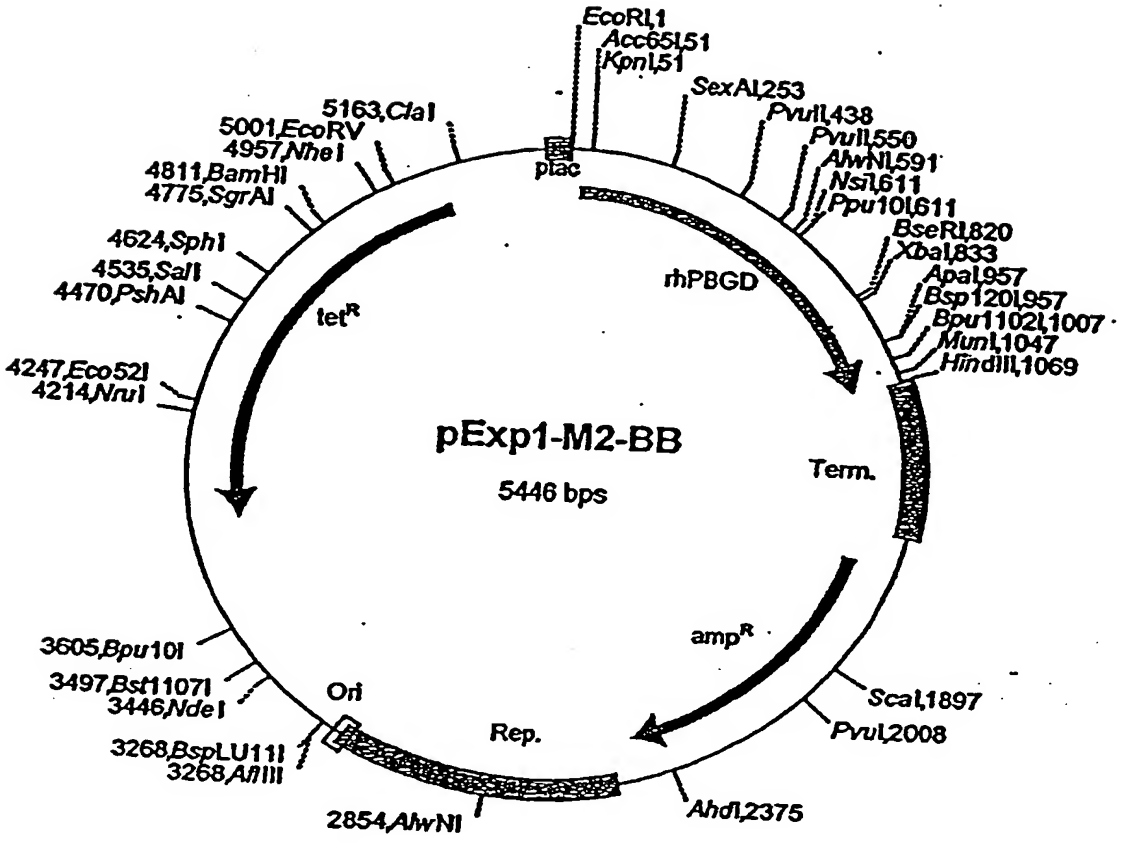

EcoRI,1
Acc65I,51
KpnI,51
ScrAI,253
NsiI,611
PpuMI,611
BseRI,820
XbaI,833
ApaI,957
BspEI,957
BpuMI,1007
MunI,1047
HindIII,1069
term.
ampR
ScaI,1897
PvuI,2008
AhdI,2375
3619,BsmBI
3521,NheI
3516,BsaAI
3497,BstBI
3446,NdeI
3268,BspLU11I
3268,AflII
4162,BpuMI
4071,BsaBI
rop
4804,Eco52I
4771,NruI
tetR
5027,PshAI
5092,ScaI
5181,SphI
5332,SgrAI
5368,BamHI
5514,NheI
5558,EcoRV
5720,ClaI
ptac
PBGD
pExp1-M2
6003 bps

Fig. 7



pExp1-M2-BB

Fig. 8



PLASMID FEATURES:

Start	End	Name
33	1067	rhPBGD (open reading frame)
1593	2453	amp^R (open reading frame)
5105	3915	tet^R (open reading frame)
5376	1	ptac (promoter)
1075	1501	Term. (terminator region)
3214	2519	Rep. (replication region)
3214		Ori (origin of replication)

Fig. 9

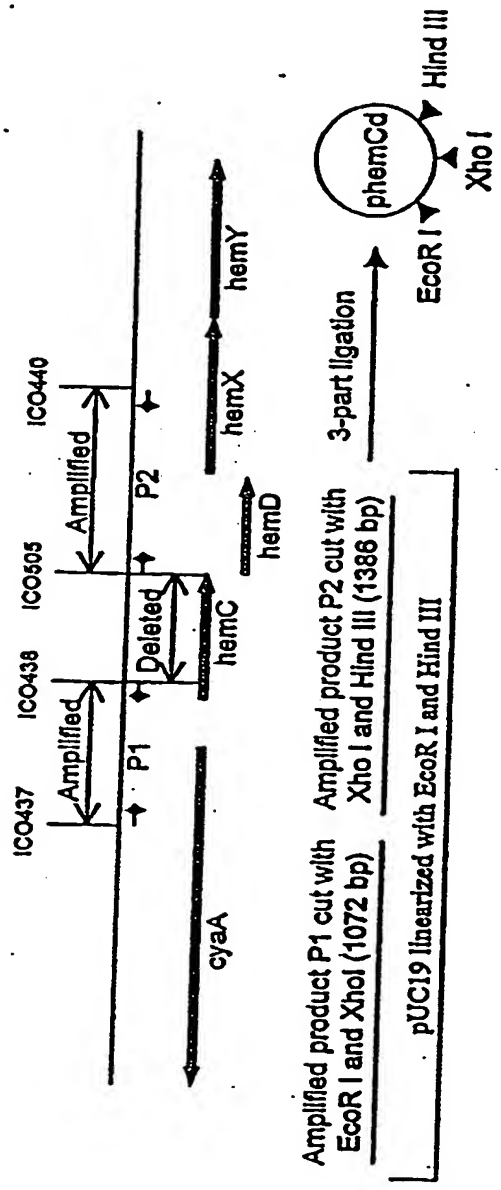

ICO437
ICO438
ICO505
ICO440
Amplified
P1
Deleted
P2
Amplified
cyaA
hemC
hemD
hemX
hemY
phemCd
Hind III
Xho I
EcoR I
3-part ligation
Amplified product P1 cut with EcoR I and XhoI (1072 bp)
Amplified product P2 cut with Xho I and Hind III (1386 bp)
pUC19 linearized with EcoR I and Hind III

Fig. 10

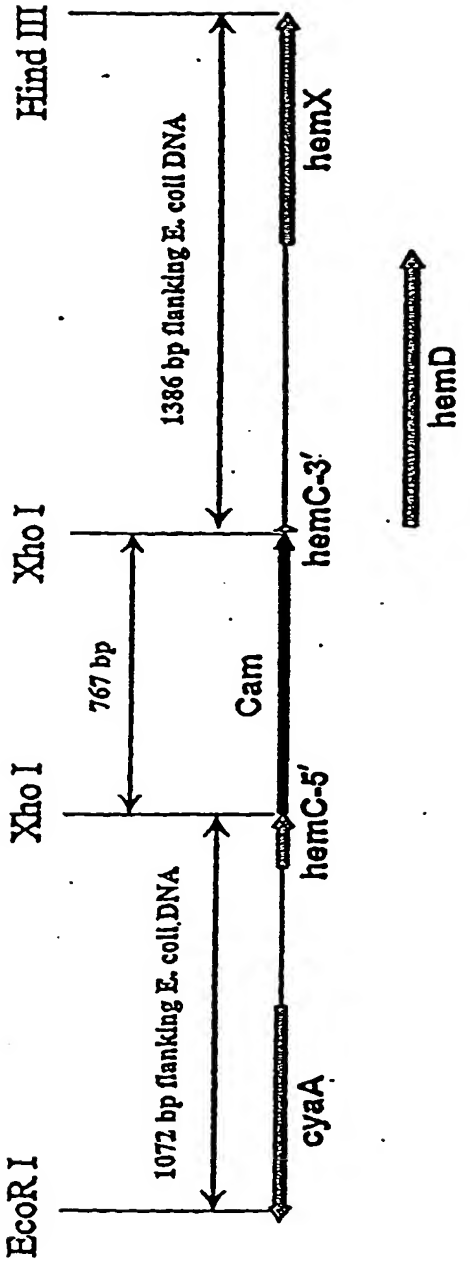

Hind III
1386 bp flanking E. coli DNA
hemX
hemD
Xho I
hemC-3'
767 bp
Cam
Xho I
hemC-5'
1072 bp flanking E. coli DNA
cyaA
EcoR I

Fig. 11

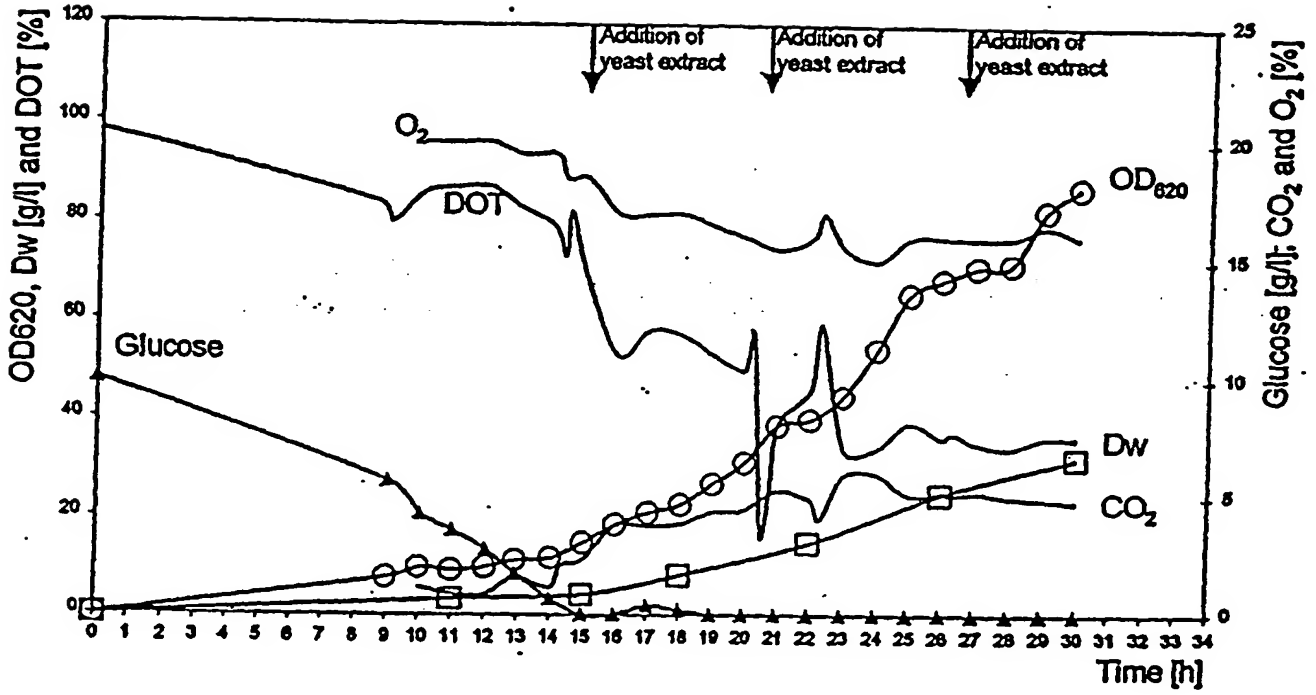

Addition of yeast extract
Addition of yeast extract
Addition of yeast extract
O2
DOT
ODG20
Glucose
Dw
CO2
OD620, Dw [g/l] and DOT [%]
Glucose [g/l]; CO2 and O2 [%]
Time [h]

Fig. 12

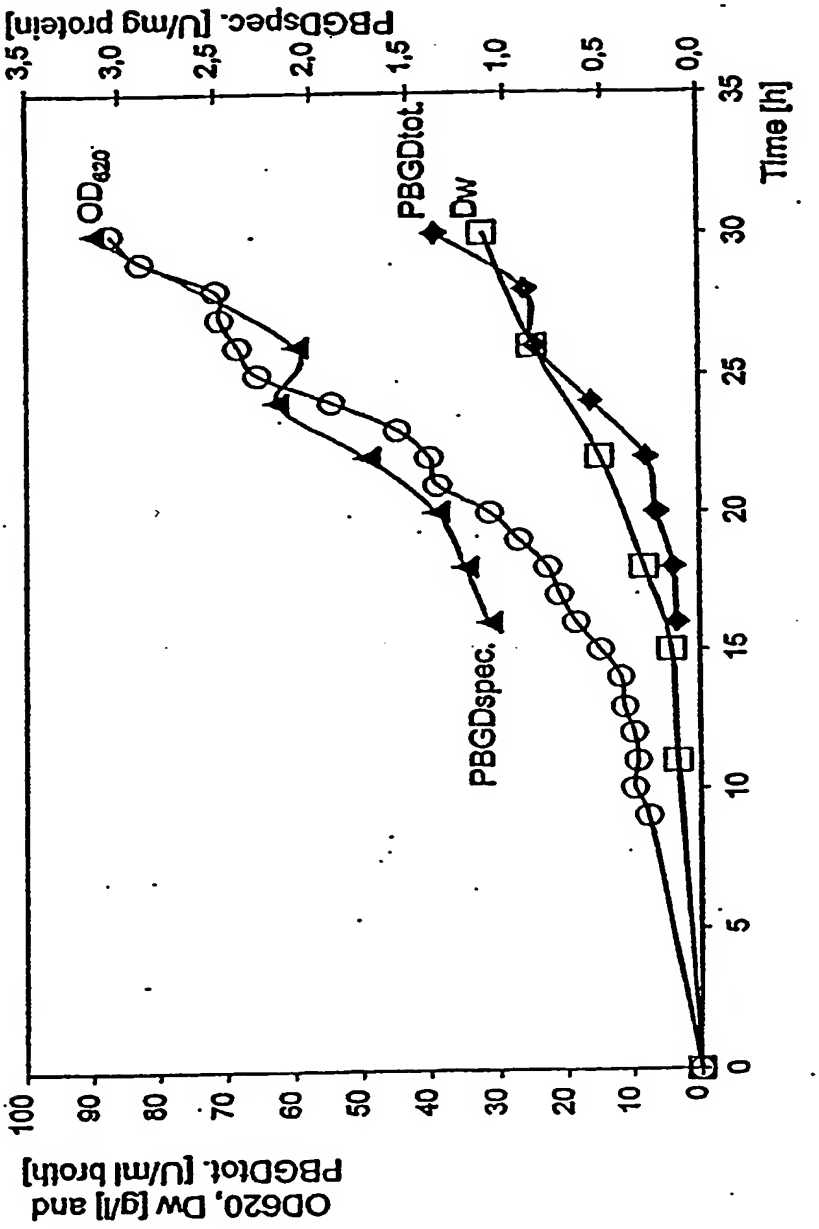
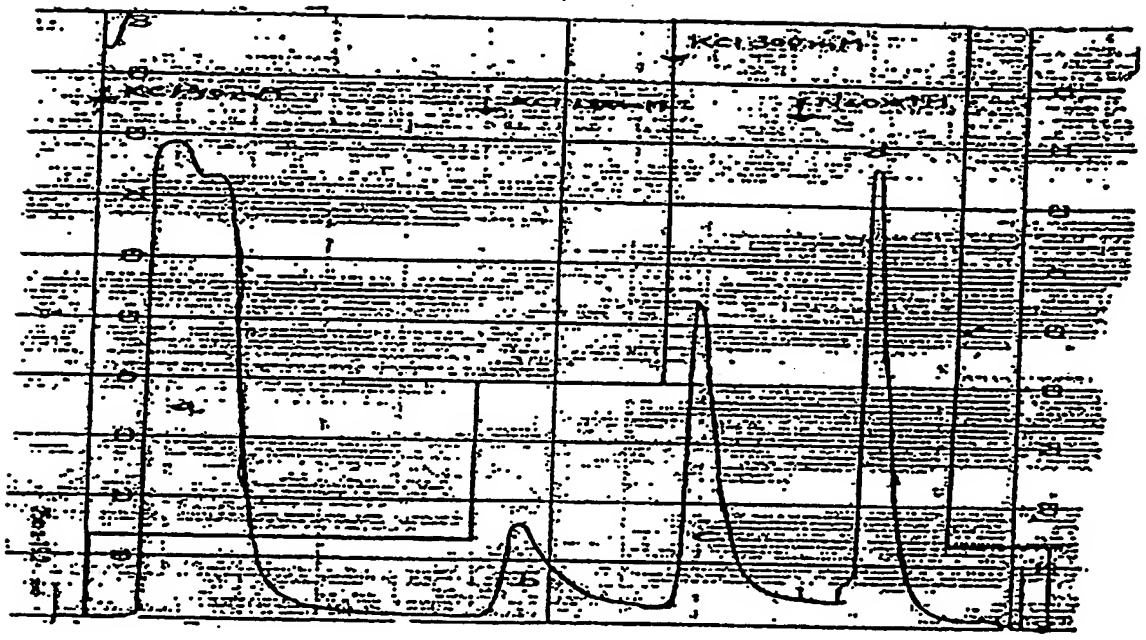

PBGDspec. [U/mg protein]
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
Time [h]
0
5
10
15
20
25
30
35
OD620, Dw [g/l] and
PBGDtot. [U/ml broth]
100
90
80
70
60
50
40
30
20
10
0
OD620
PBGDtot.
Dw
PBGDspec.

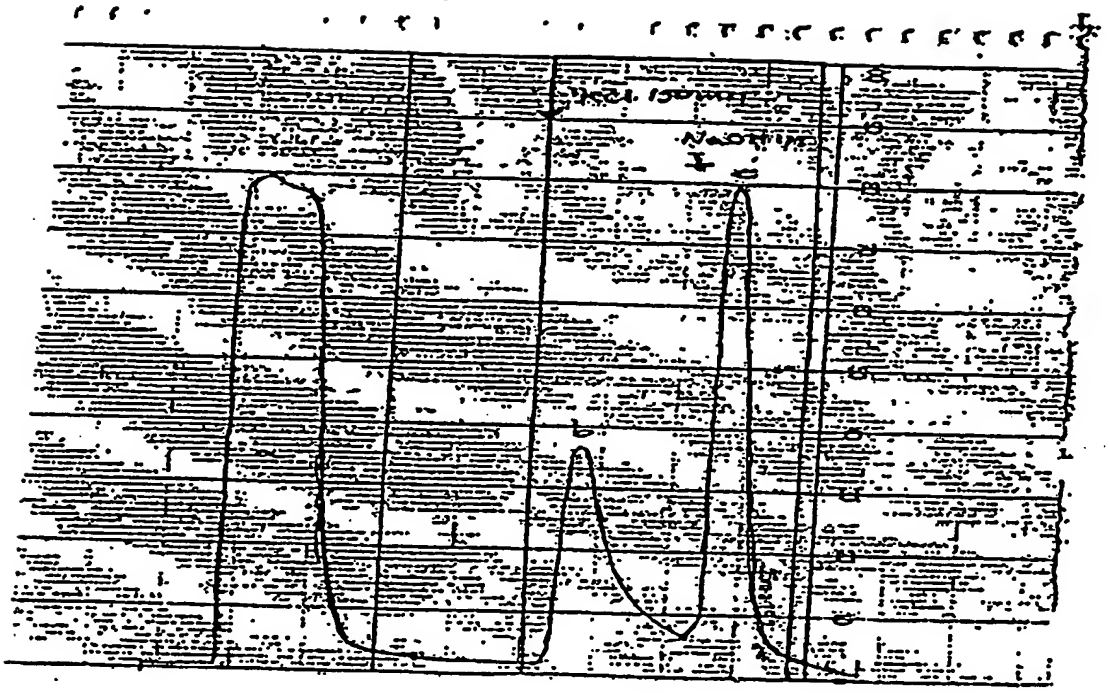
Fig. 13

Fig. 13



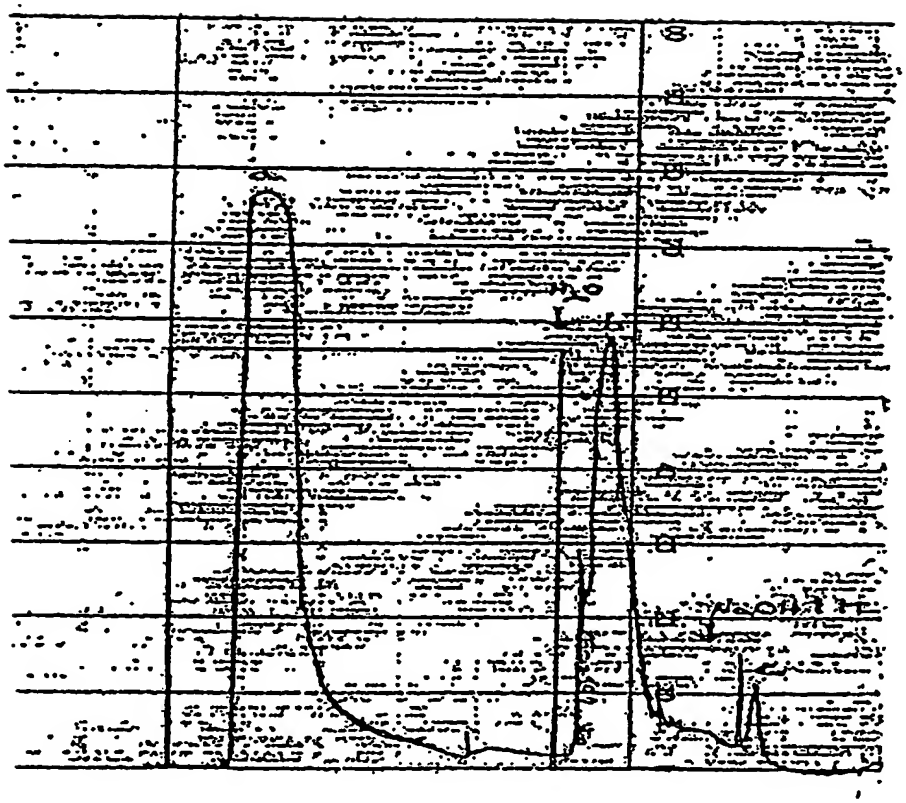
Chromatography on DEAE-Sepharose FF (DEAE1). Peak a flow through the gel 40 mM KCl, Peak b 120 mM KCl Peak c 300 mM KCl Peak d NaOH 1 M.

Fig. 14



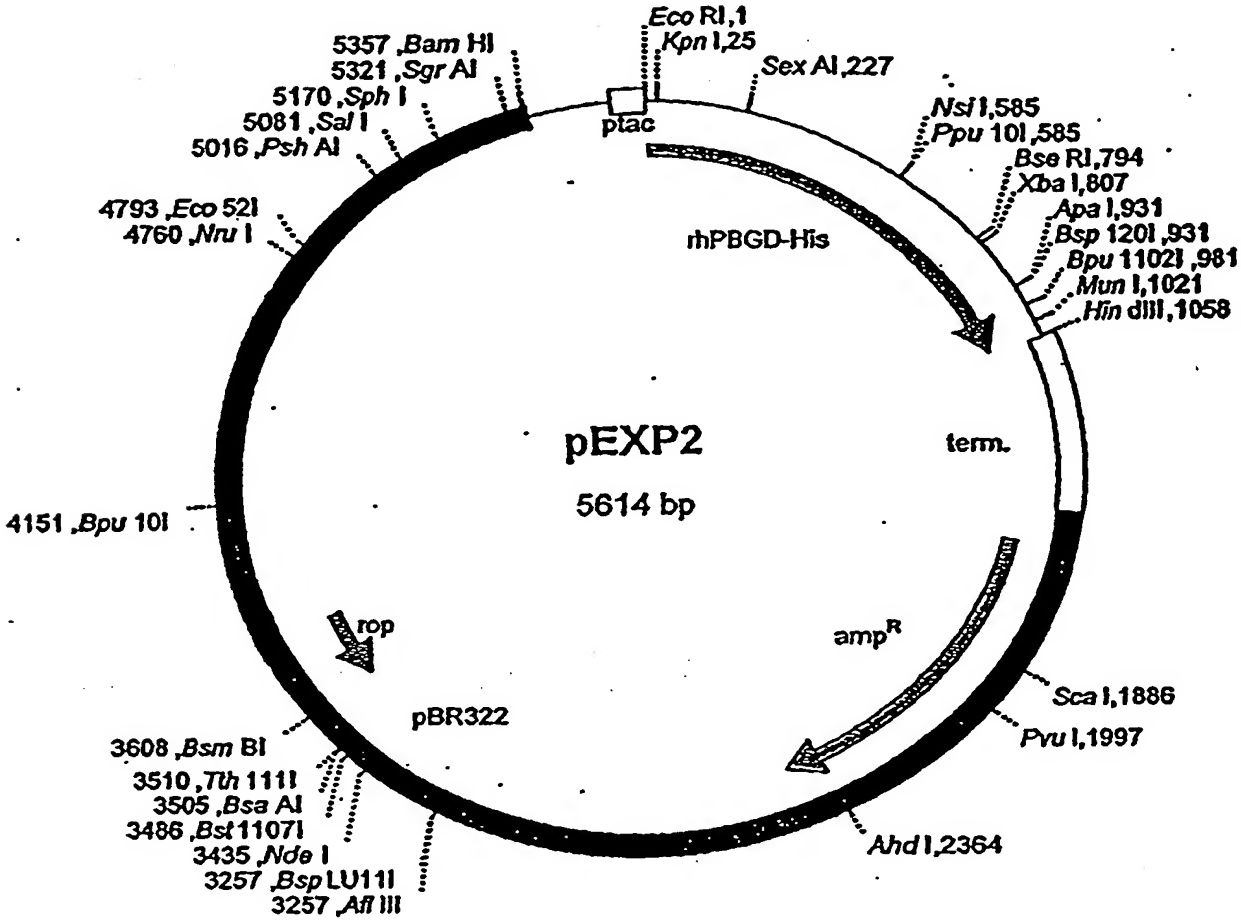
Chromatography on DEAE-Sepharose FF (DEAE2). Peak a flow through the gel 40 mM KCl, Peak b 150 mM KCl Peak c NaOH 1 M.

Fig. 15



Chromatography on Butyl-Sepharose 4 FF. Peak a flow through the gel, Peak b water Peak c NaOH 1 M.

Fig. 16



PLASMID FEATURES:

Start	End	Name
7	1056	rhPBGD-His
1064	1490	terminator region
1582	2442	amp^R
3820	3629	rop
5362	1490	pBR322
5544	1	ptac (promoter)

Fig. 17

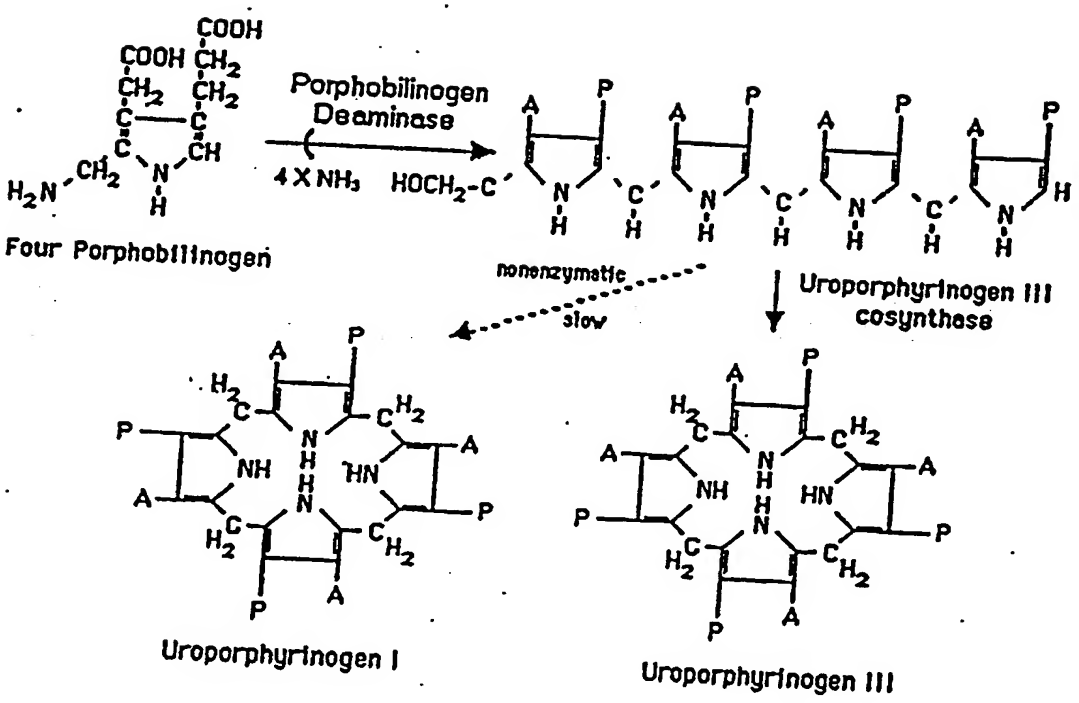

Porphobilinogen Deaminase
4 X NH3
Four Porphobilinogen
nonenzymatic
slow
Uroporphyrinogen III cosynthase
Uroporphyrinogen I
Uroporphyrinogen III

Fig. 18

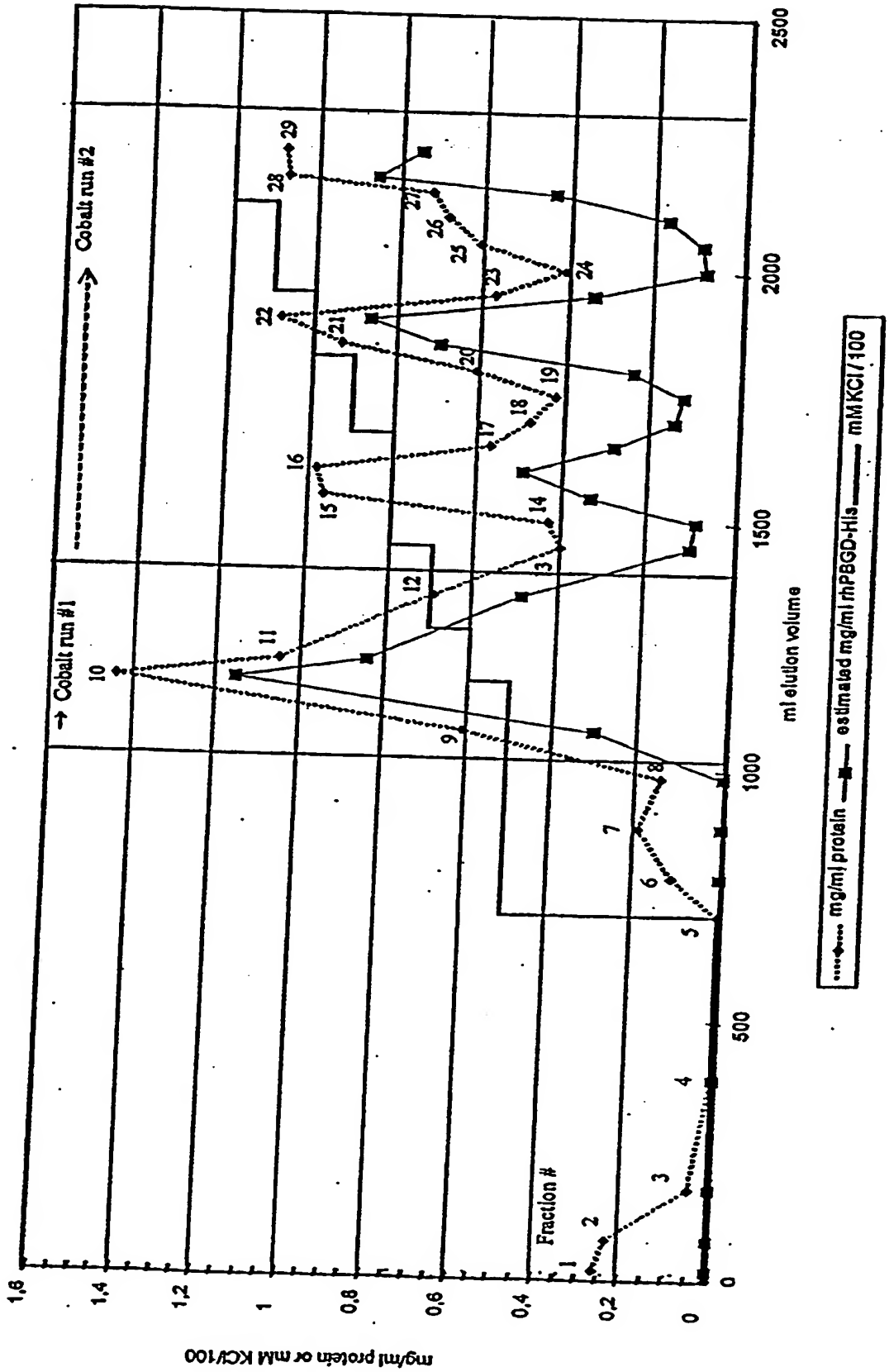
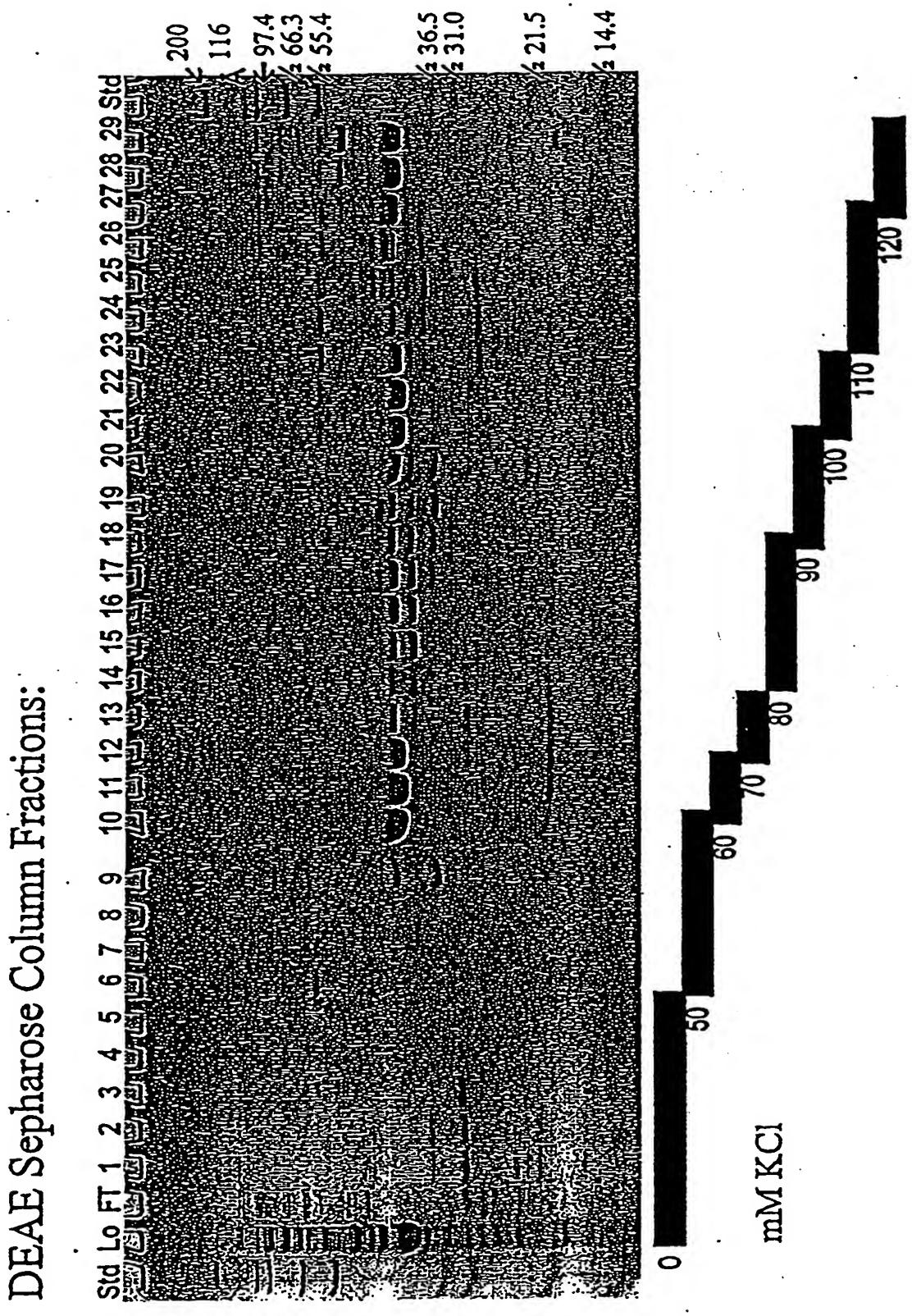

Cobalt run #1
Cobalt run #2
Fraction #
ml elution volume
mg/ml protein or mM KCl/100
mg/ml protein
estimated mg/ml rhPBGD-His
mM KCl / 100
0
500
1000
1500
2000
2500
0,2
0,4
0,6
0,8
1
1,2
1,4
1,8

Fig. 19


DEAE Sepharose Column Fractions:
Std Lo FT 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 Std
200
116
97.4
66.3
55.4
36.5
31.0
21.5
14.4
0
50
60
70
80
90
100
110
120
mM KCl

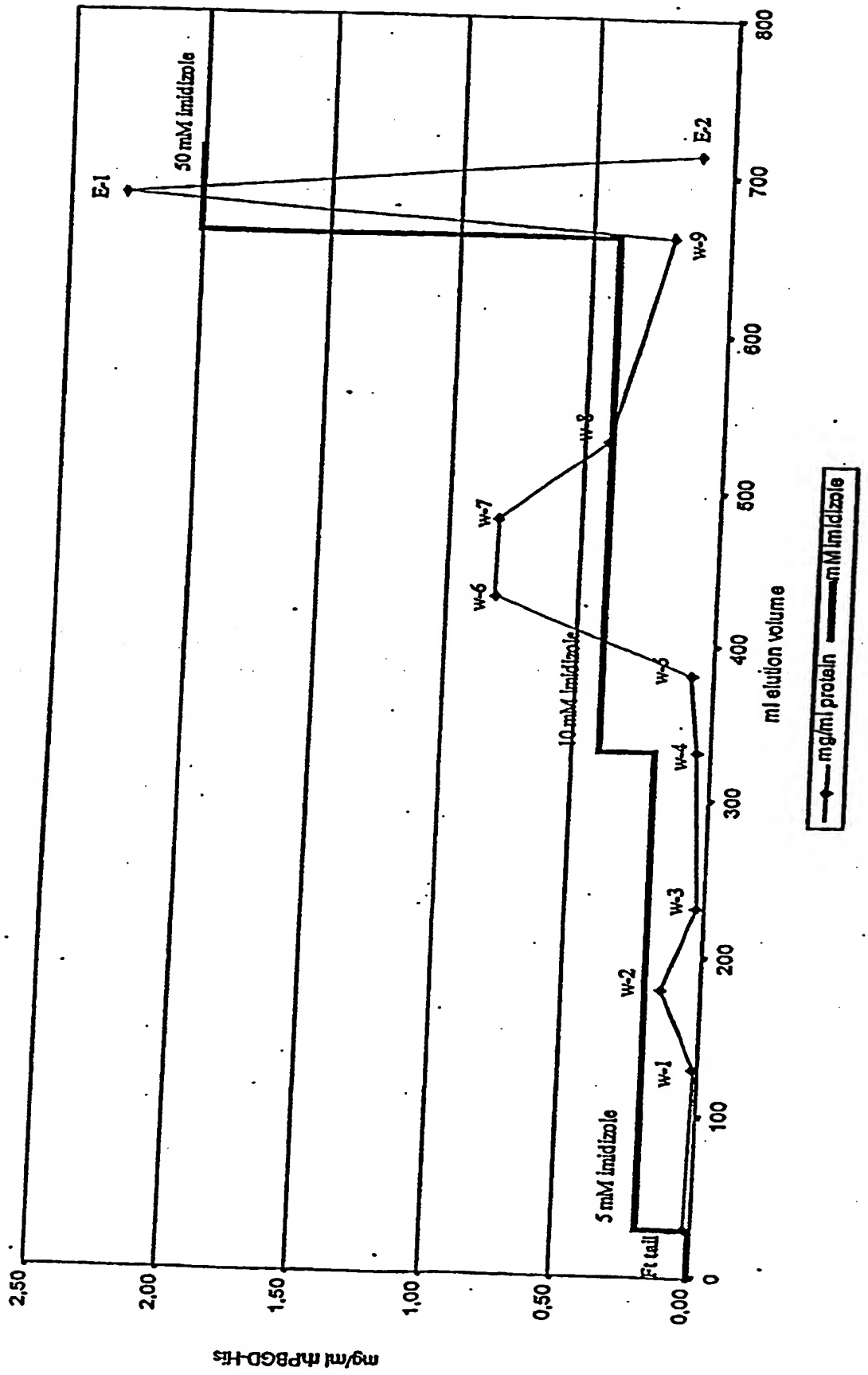

50 mM imidizole
E-1
E-2
w-9
w-8
w-7
w-6
w-5
10 mM imidizole
w-4
w-3
w-2
w-1
5 mM imidizole
Ft tail
2,50
2,00
1,50
1,00
0,50
0,00
800
700
600
500
400
300
200
100
0
ml elution volume
mg/ml protein
mM imidizole
mg/ml rhPBGD-His

Fig. 21

1	Mark 12 molecular weight standards (Novex)
2	Cobalt load #1 (DEAE fractions 9 -> 12)
3	Flowthrough from cobalt load #1
4	50 mM imidizole eluate from cobalt run #1
5	Concatamerized nickel purified rhPBGD-His
6	Flowthrough from cobalt load #2
7	10 mM imidizole eluate from cobalt run #2
8	50 mM imidizole eluate from cobalt run #2

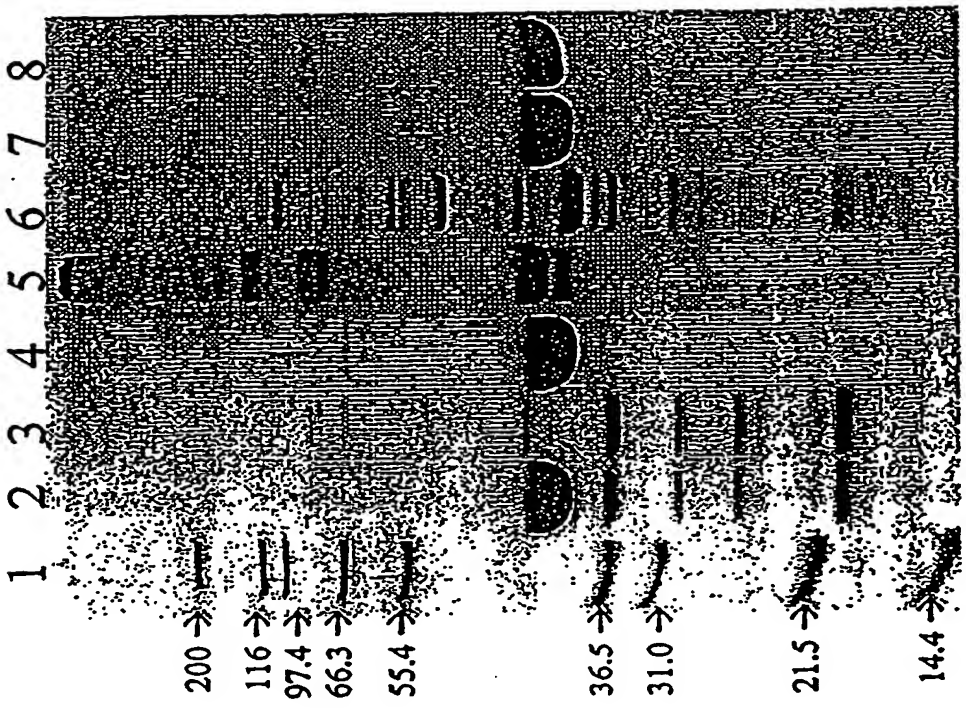


Fig. 22

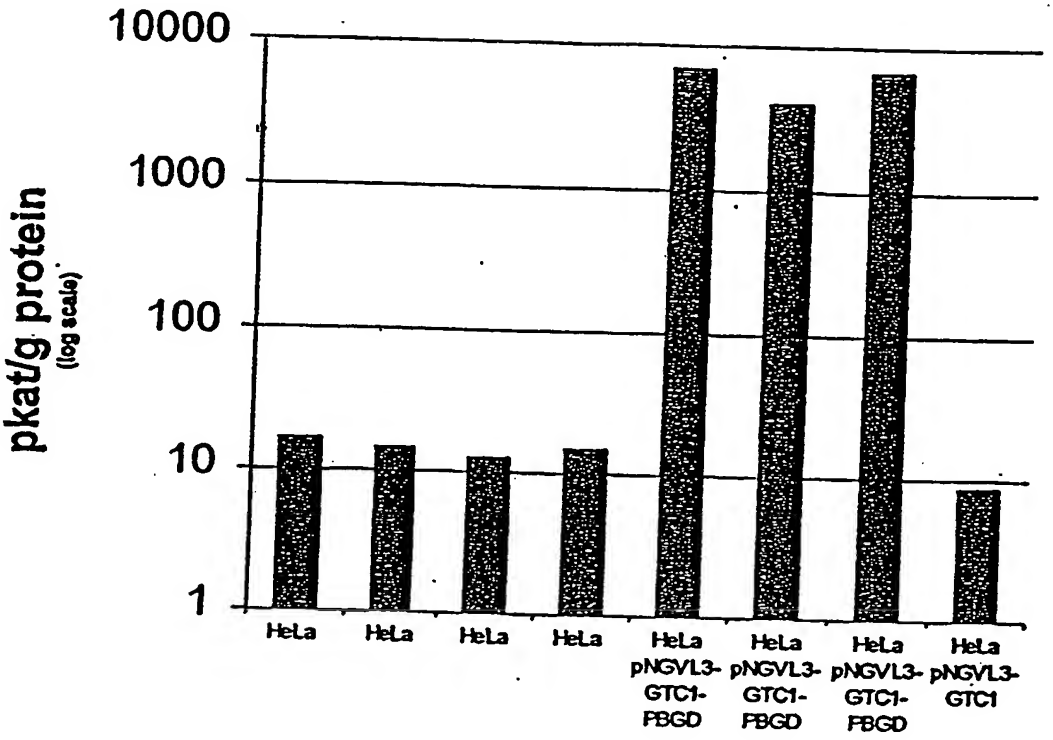


Fig 23: PBGD activity related to protein concentration in HeLa cells.

Fig. 23

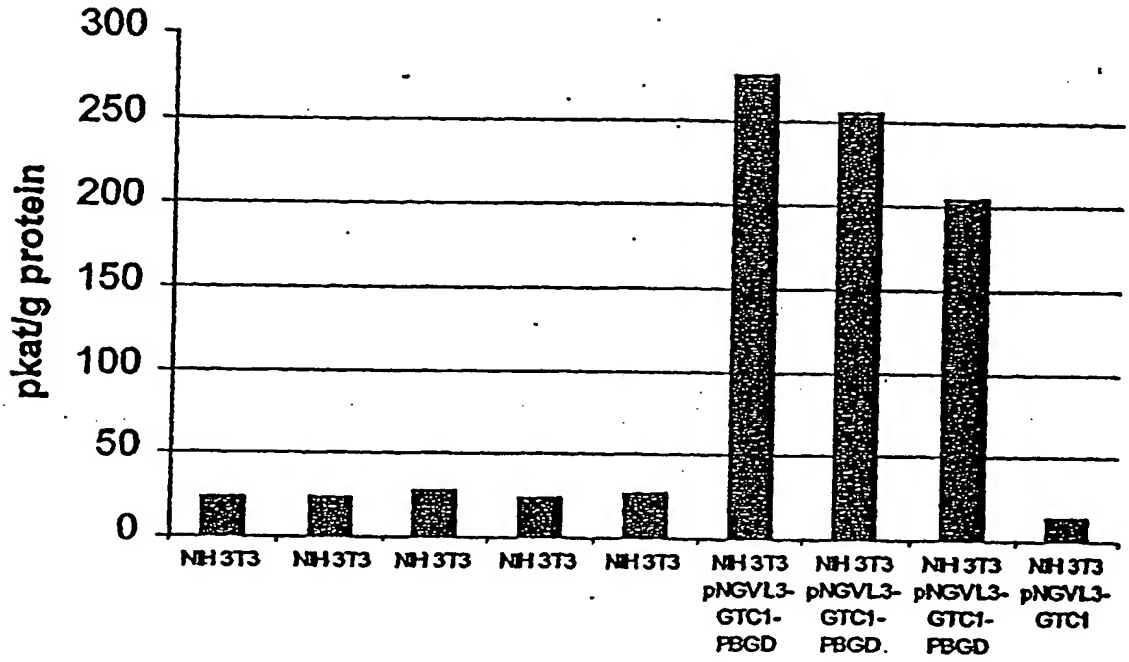


Figure 24: PBGD activity related to protein concentration in NIH 3T3 cells.

Fig. 24

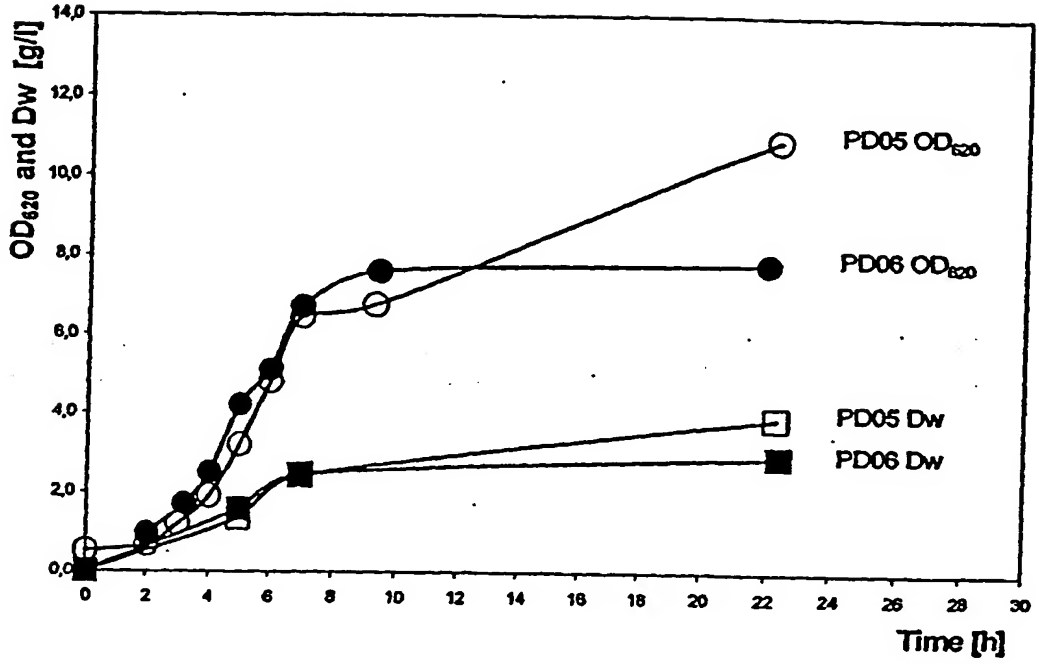


Fig 25. Comparison of fermentations PD05 and PD06 with strain PBGD-2.

Fig. 25

Fig 26. Comparison of fermentations PD09, PD11 and PD12

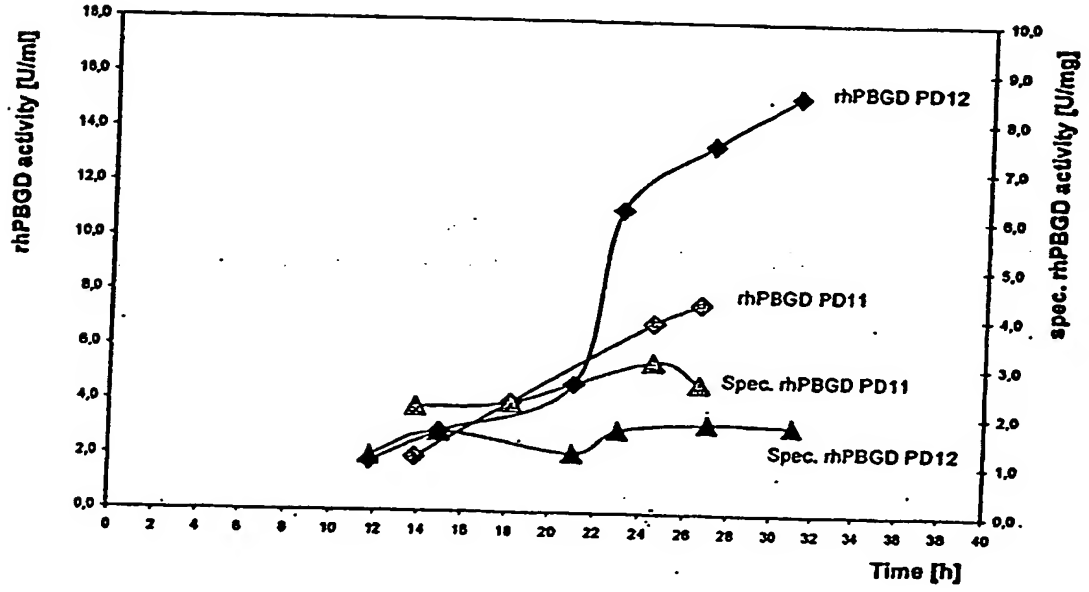


Fig. 26

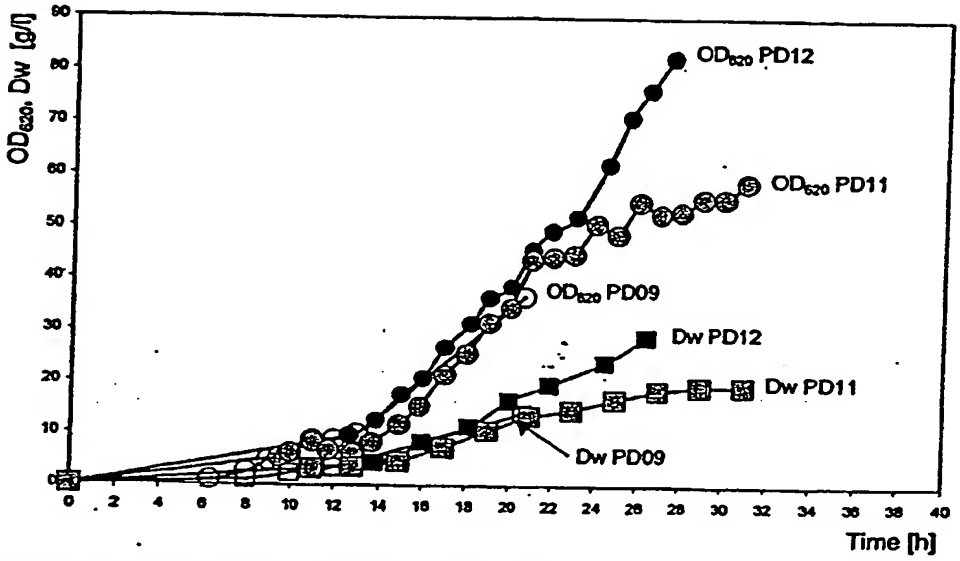


Fig 27: Comparison of fermentations PD09, PD11 and PD12 with strain PBGD-1.

Fig. 27

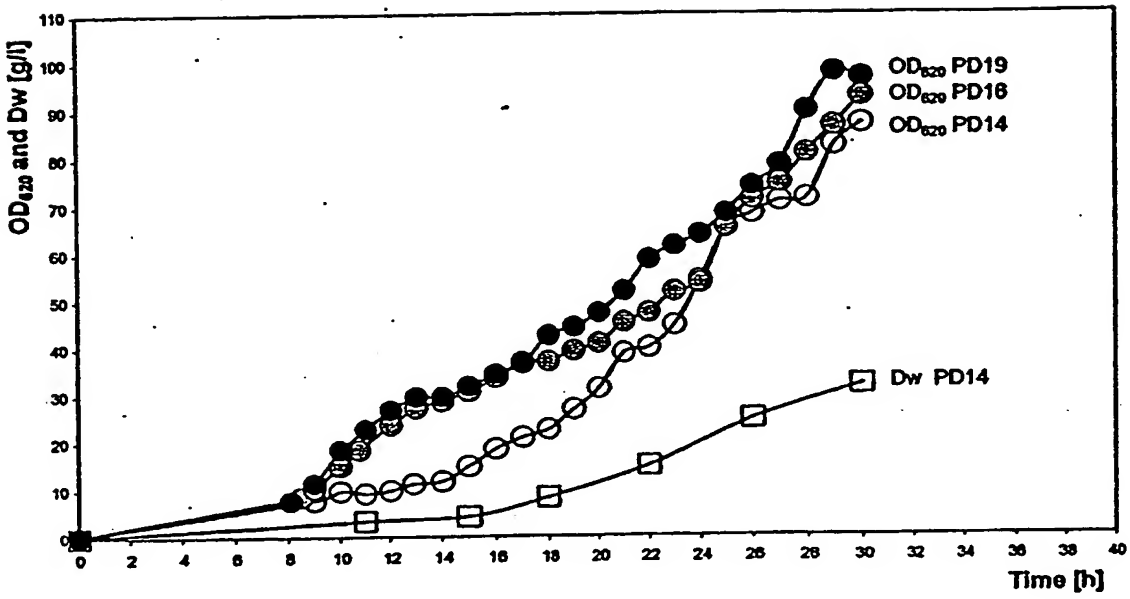


Fig 28.Comparison of fermentations PD14, PD16 and PD19 with strain PBGD-2.

Fig. 28

Fig 29.Comparison of fermentations PD14, PD16 and PD19 with strain PBGD-2.

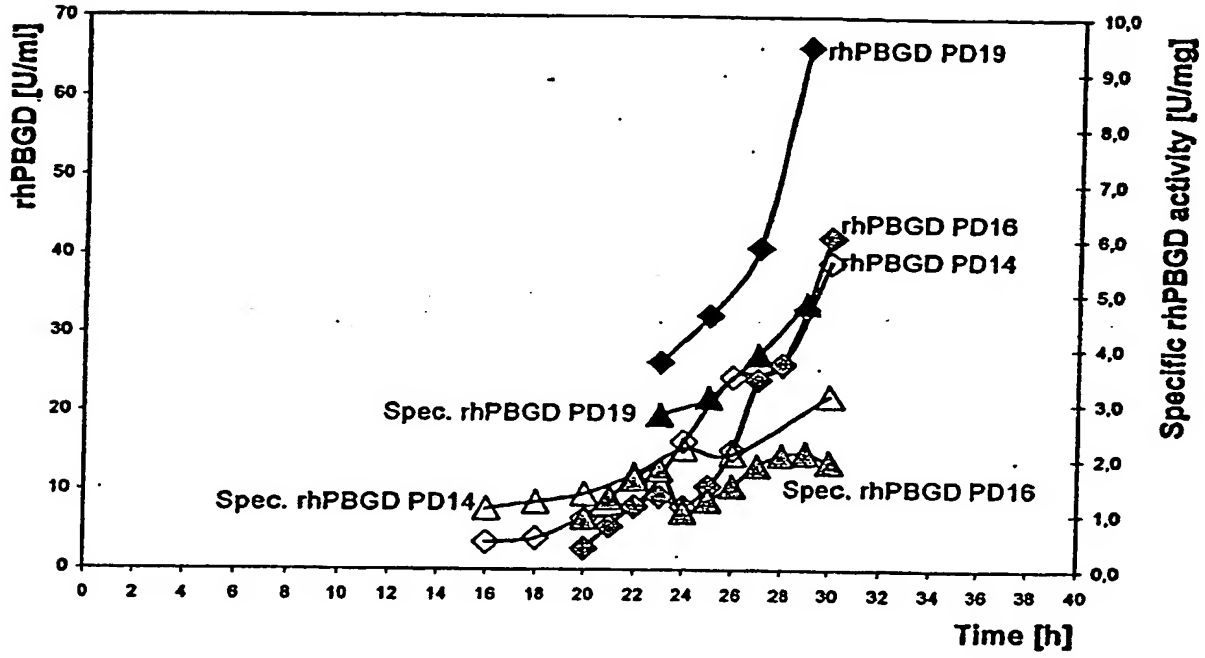


Fig. 29

Fig 30.Comparison of fermentations PD19, PD21 and PD22 with strain PBGD-2.

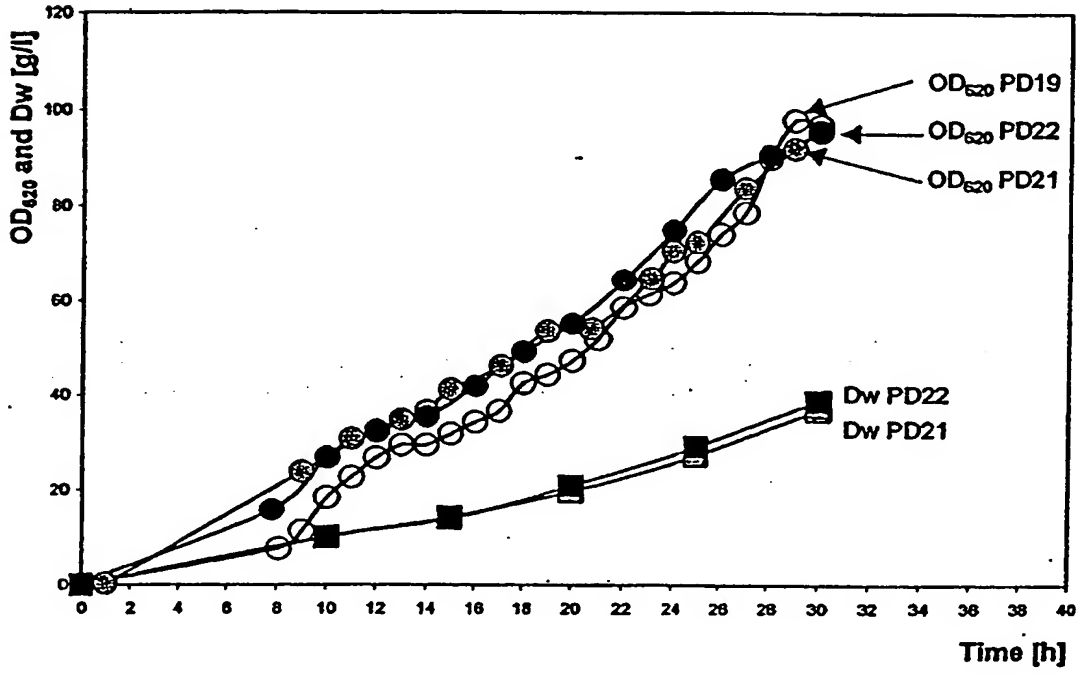


Fig. 30

Fig 31. Comparison of fermentations PD19, PD21 and PD22 with strain PBGD-2.

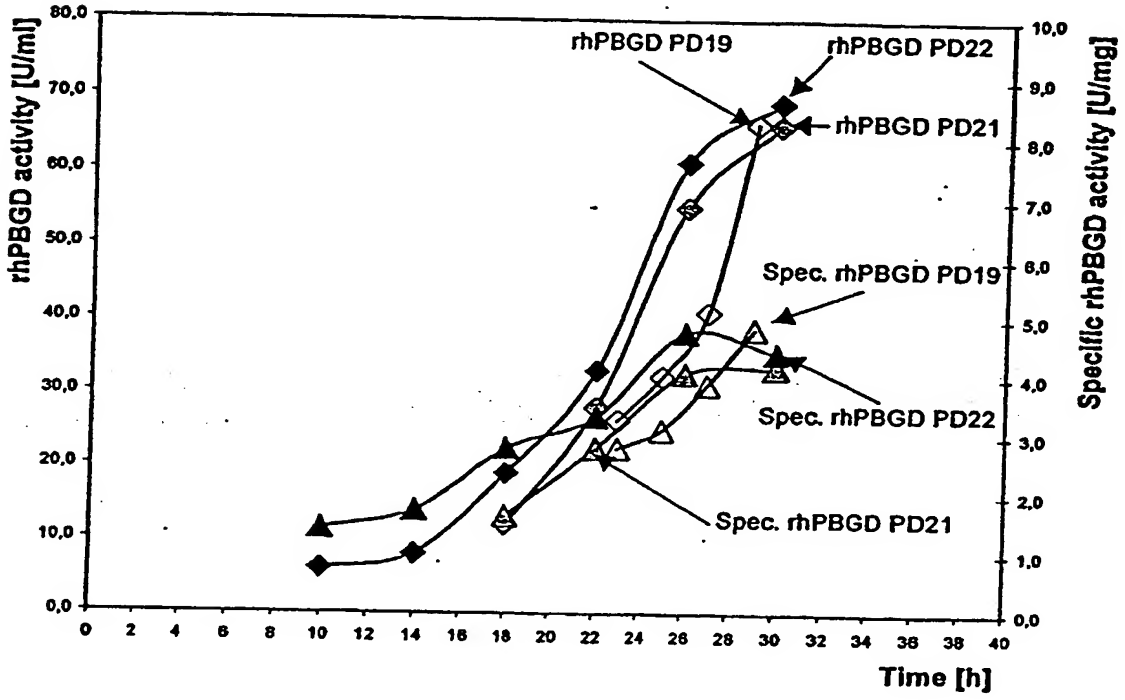


Fig. 31

Fig 32. Comparison of fermentations PD19, PD1501 and PD1502.

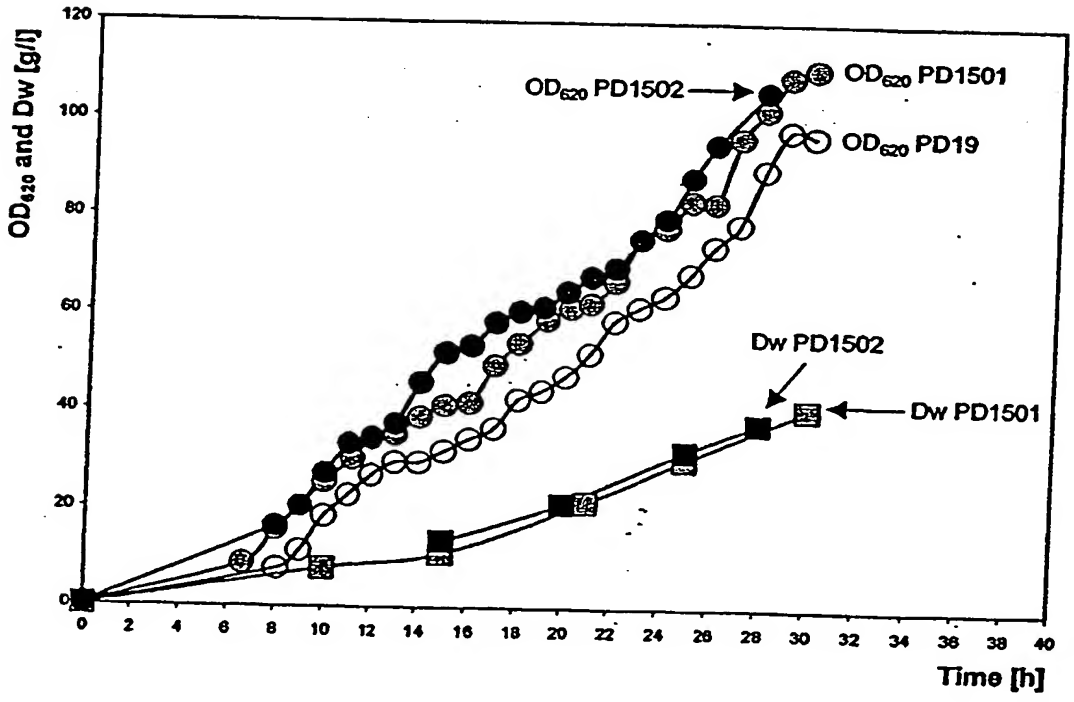


Fig. 32

Fig 33. Comparison of fermentations PD19, PD1501 and PD1502 with strain PBGD-2.

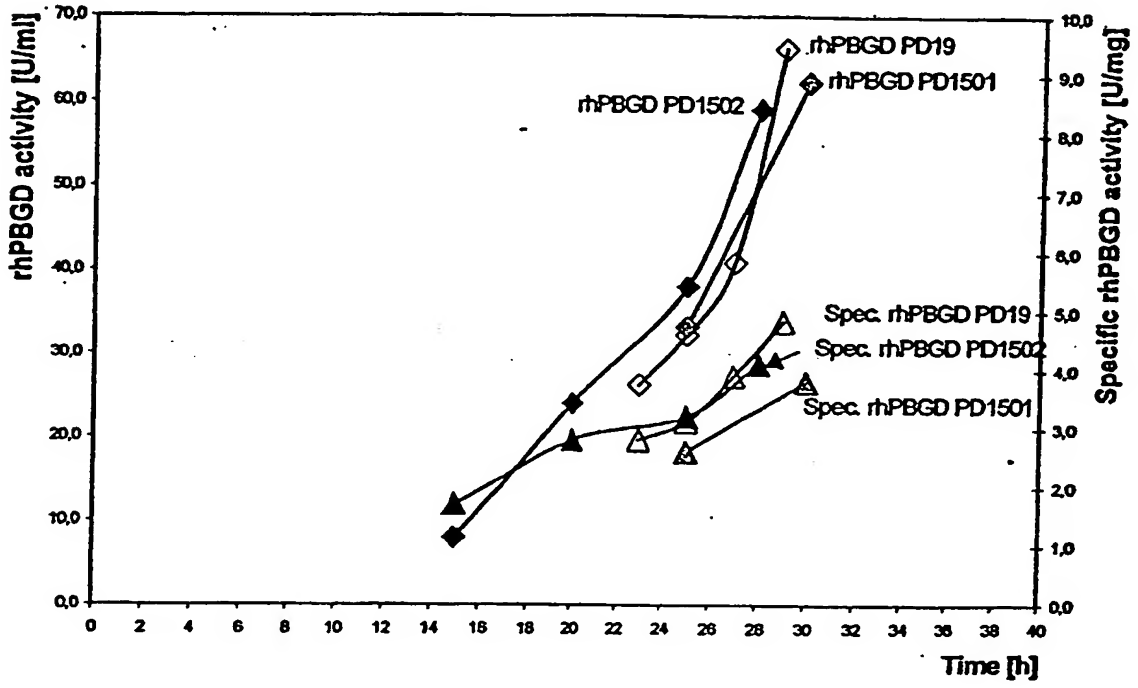
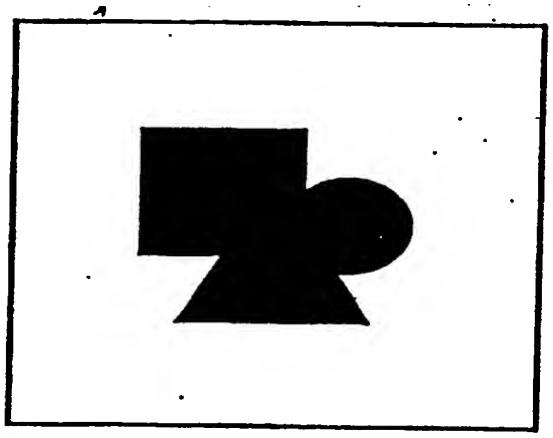
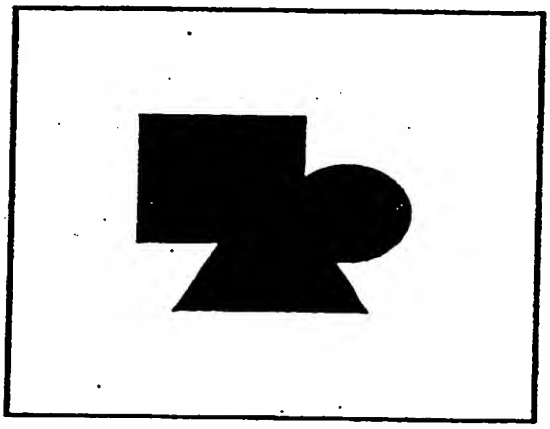


Fig. 33

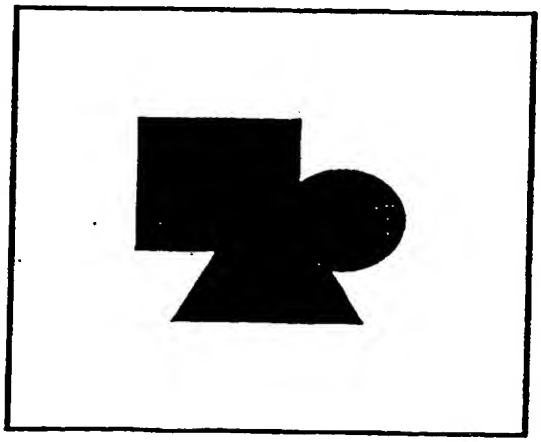
Summary of Fermentation and down stream process visualized by SDS-Page. Comparison between different samples.



Well	Samples from PD22
1	Mark 12 MW st
2	Extract
3	Homogenate
4	Broth 30 h
5	Broth 26 h
6	Broth 22 h
7	Broth 18 h
8	Broth 14 h
9	Broth 10 h
10	rhPBGD-His



	Samples from PD1501
1	Mark 12 MW st
2	Extract
3	Extract
4	Extract
5	Broth 30 h
6	Broth 30 h
7	Broth 25 h
8	Broth 20 h
9	Broth 15 h
10	rhPBGD-His



	Samples from PD1502
1	Mark 12 MW st
2	Broth 15 h
3	Broth 20
4	Broth 25 h
5	Broth 28 h
6	Homogenate
7	Extract
8	Extract
9	Extract
10	rhPBGD-His

Fig. 34

Fig 35. Stability studies: Single use aliquots of extract were routinely taken out of the freezer (-20°C) and the rhPBGD-activity was measured and plotted over time.

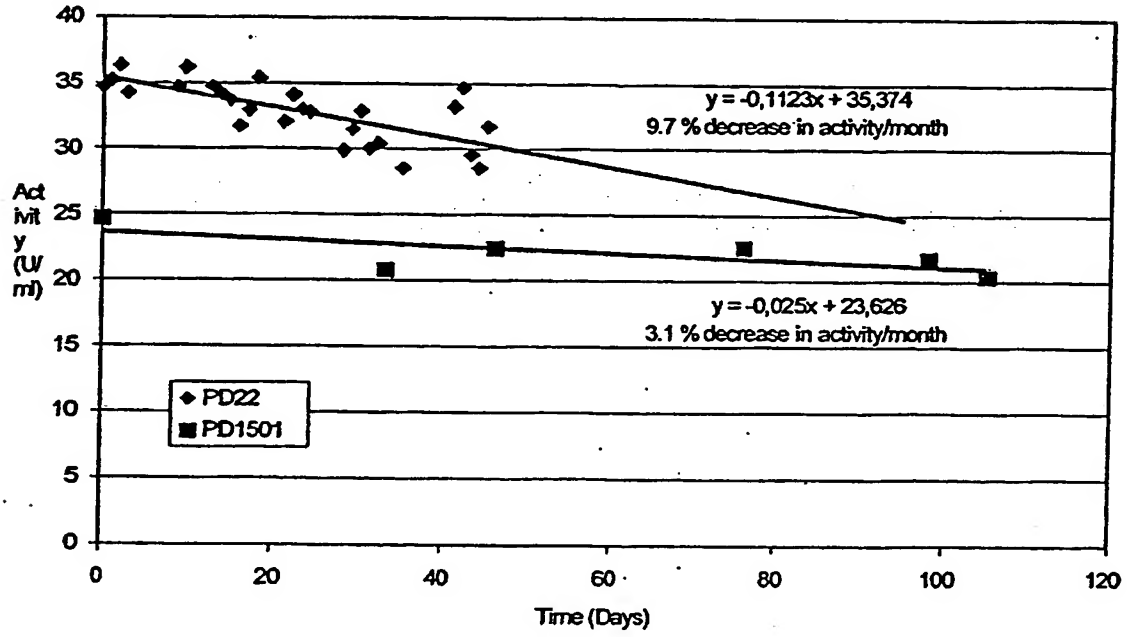
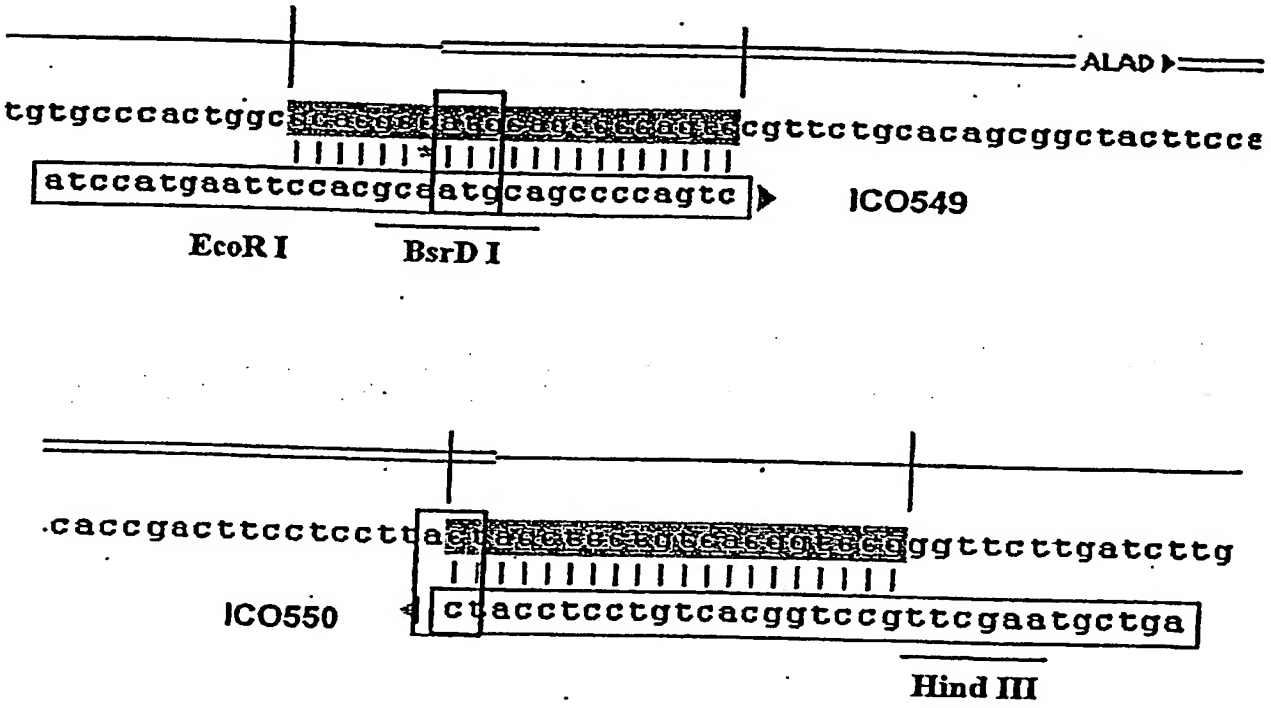


Fig. 35

Description of the oligos used for PCR amplification



The two oligos ICO549 and ICO550 are shown below the ALAD sequence. The highlighted regions in the ALAD sequence represent the regions of homology with the oligos. The asterisk denotes the C to A change introduced to generate a *Bsr*D I site for ease of manipulation of the 5 ' end of the cloned molecule. The initiation and termination triplets in the ALAD sequence are boxed. Both the oligos have 5 ' sequences extending beyond the regions of homology to incorporate restriction sites for cloning of the amplified DNA, *viz. Eco*R I for ICO549 and *Hin*d III for ICO550.

Fig. 36

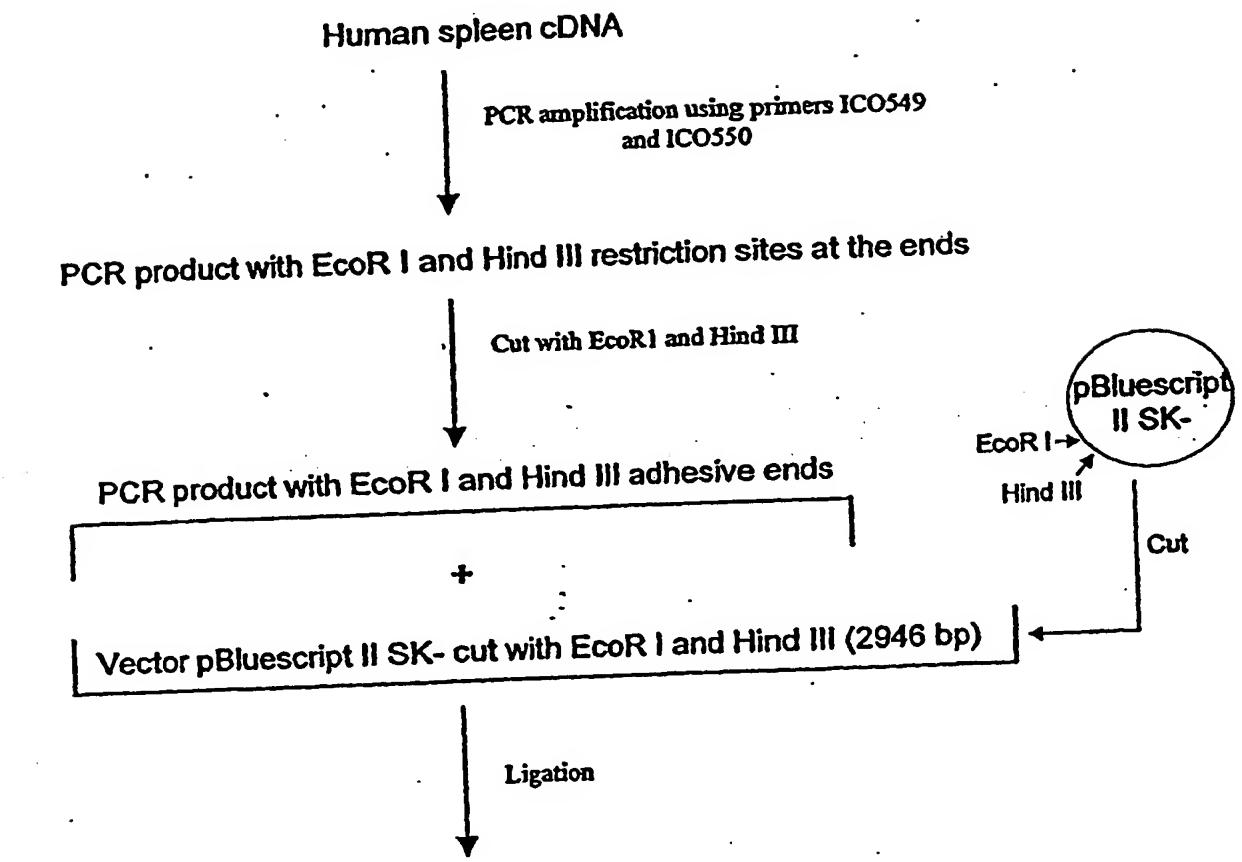

Strategy for PCR cloning of ALAD
Human spleen cDNA
PCR amplification using primers ICO549 and ICO550
PCR product with EcoR I and Hind III restriction sites at the ends
Cut with EcoR1 and Hind III
pBluescript II SK-
EcoR I
Hind III
Cut
PCR product with EcoR I and Hind III adhesive ends
+
Vector pBluescript II SK- cut with EcoR I and Hind III (2946 bp)
Ligation

Fig. 37

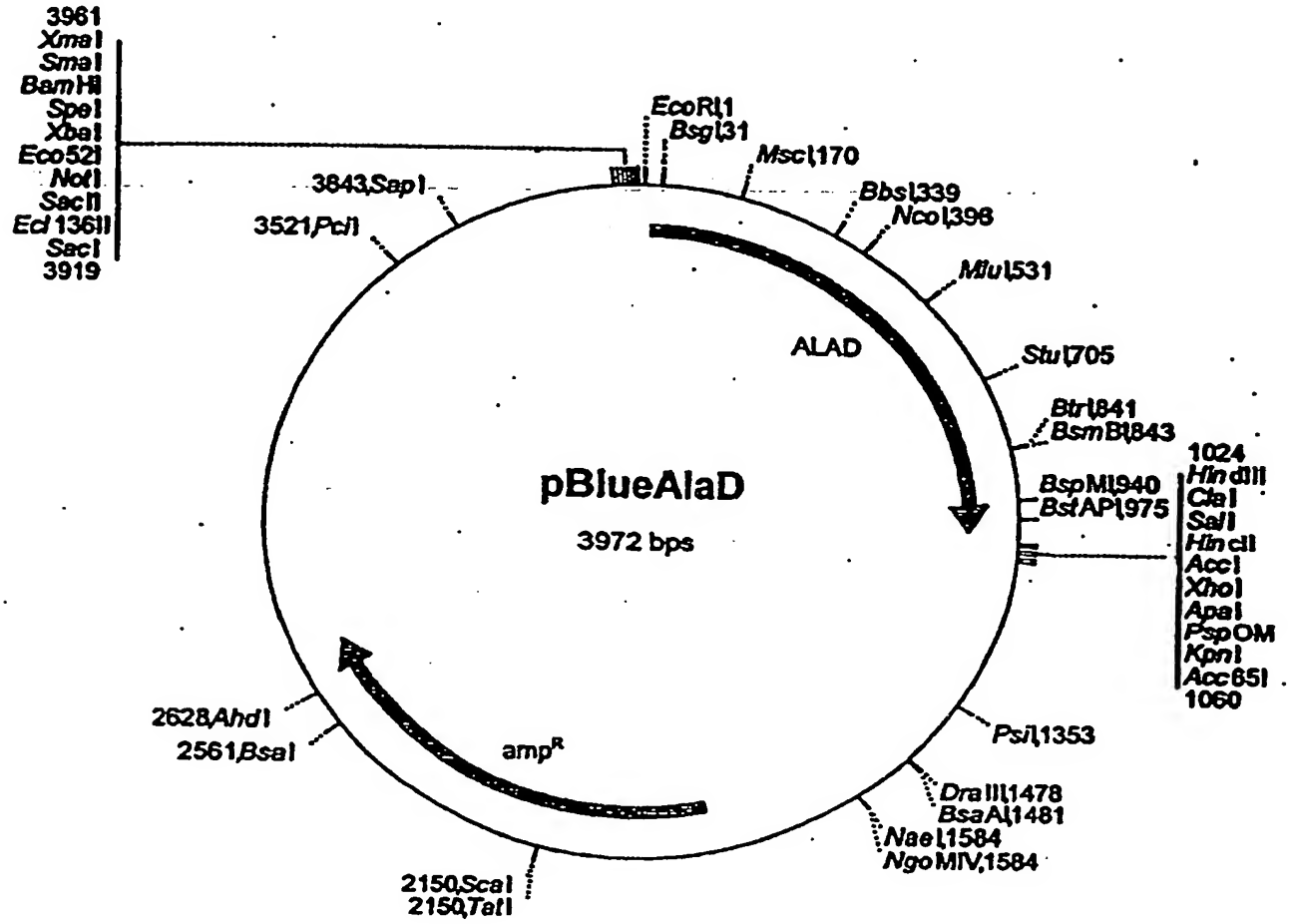


Fig. ~~38~~ 37C

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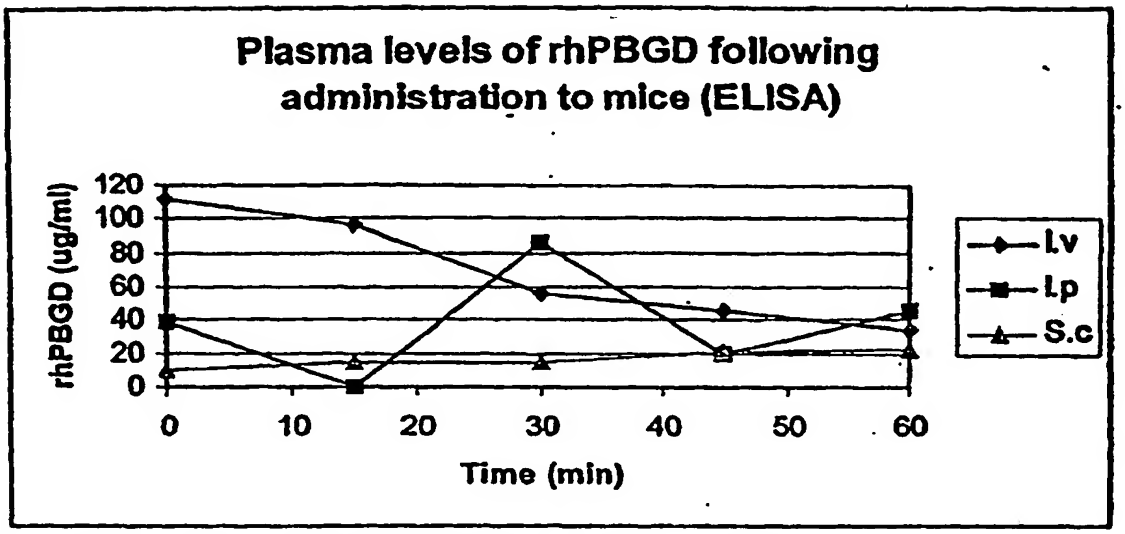

Figure 38: Plasma levels of rhPBGD following administration to mice.

~~Fig. 39~~

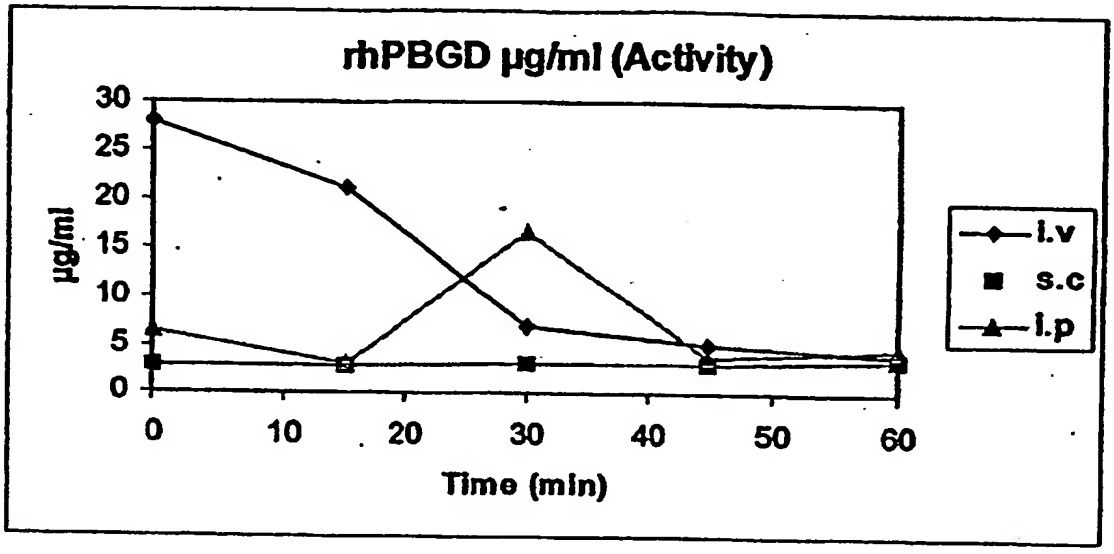

Figure 39: PBGD enzymatic activity in plasma following rhPBGD administration to mice

Fig. 40

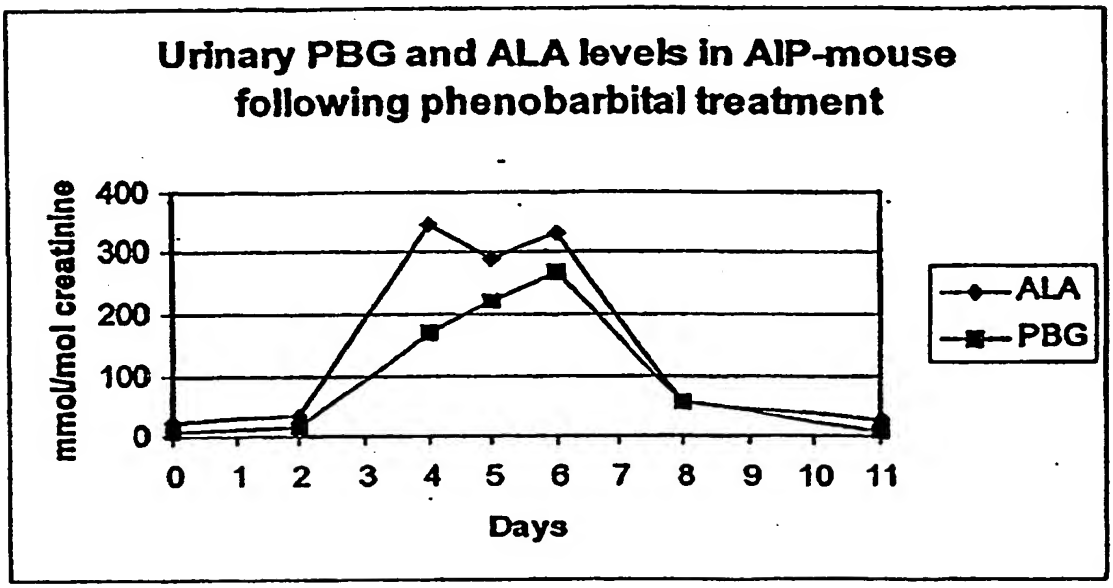

Figure 40: Urinary content of PBG and ALA in AIP-mouse treated with phenobarbital.

~~Fig. 41~~

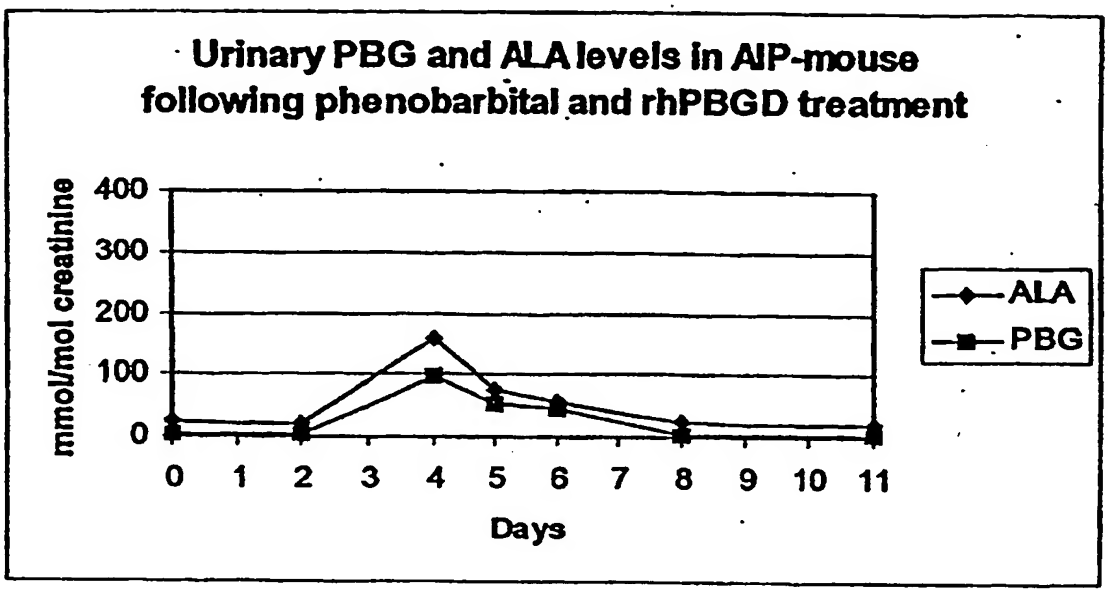

Figure 41: Urinary content of PBG and ALA in AIP-mouse treated with phenobarbital and rhPBGD. Mice were treated with an increasing dose of phenobarbital for 4 days (day 0-4,

Fig. 42

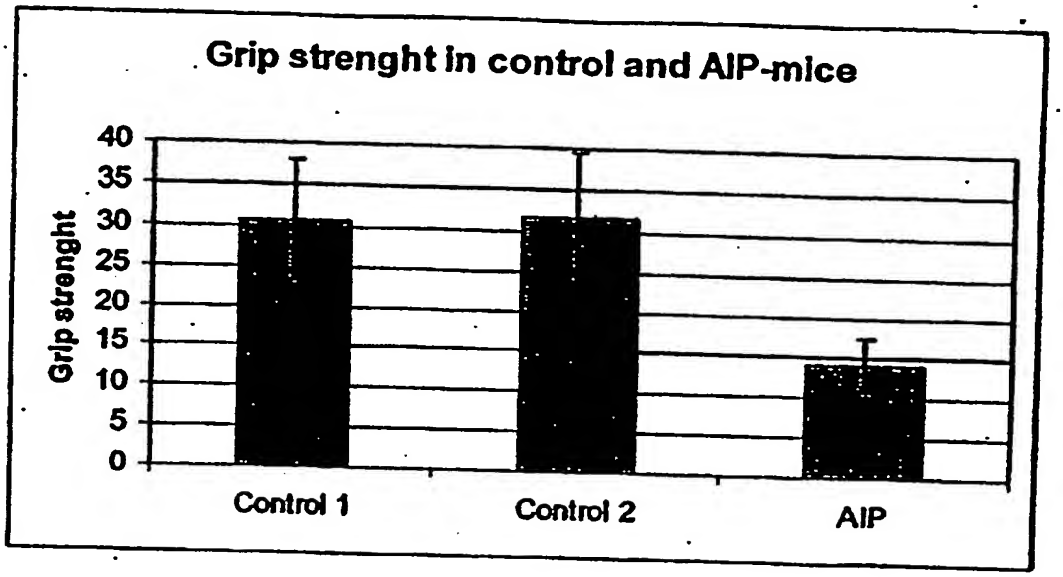

Figure 42: Grip strength analysis in control and AIP-mice. Grip strength were determined using a grip strength meter (Ugo Basile) in heterozygous control animals (control 1, n=5), in wild type controls (control 2, n=5) and in AIP-transgenic mice (AIP, n=5).

Fig. 43

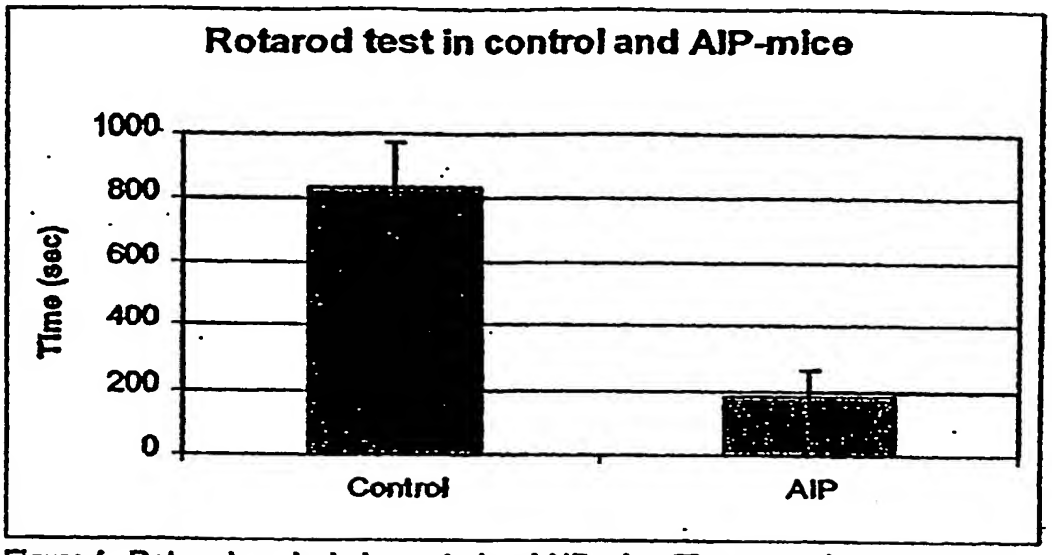

Figure 4 Rotarod analysis in control and AIP-mice. The rotarod analysis were determined using a rotarod treadmill (Ugo Basile) in wild type controls (control, n=5) and in AIP-transgenic mice (AIP, n=7).

Fig. ~~44~~ 43

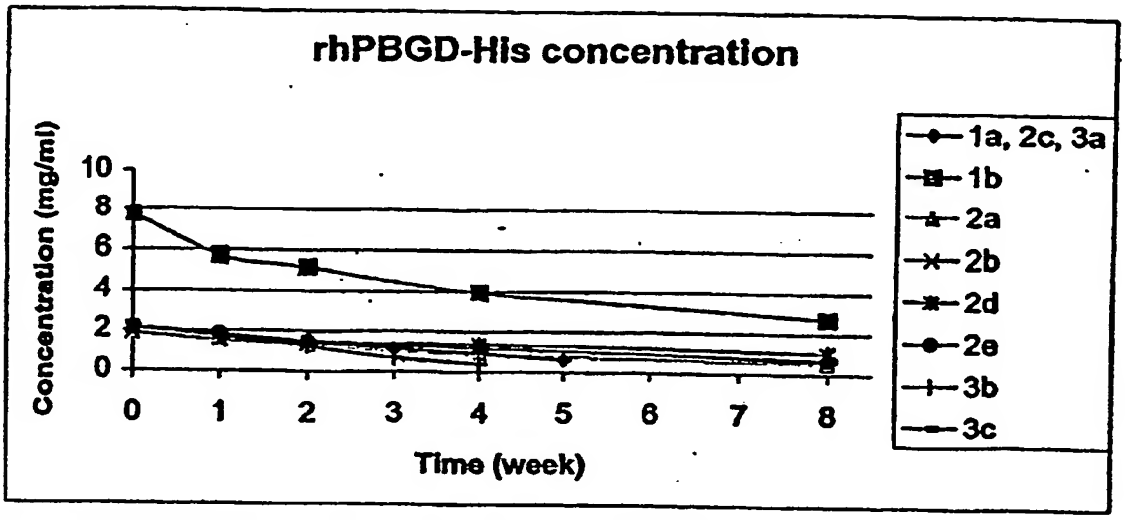

Figure 44. Enzyme concentration over 8 weeks at 40°C measured by HPLC. A decrease from 2 mg/ml to 0,5 mg/ml and 8 mg/ml to 2,5 was detected.

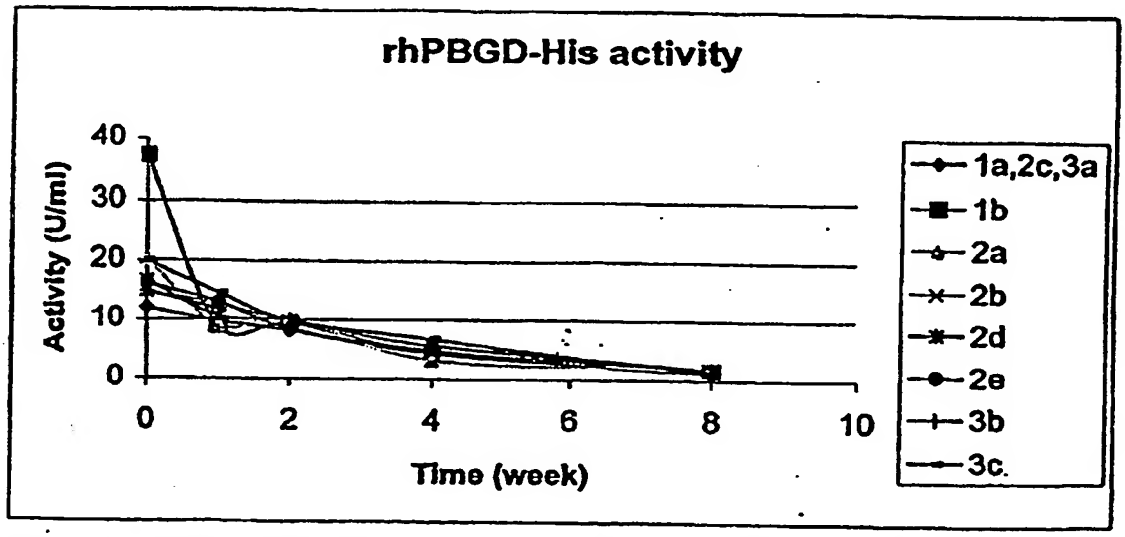

Figure 45. The enzyme activity measured over 8 weeks at 40°C. A significant decrease over the first week was seen for the high concentration sample, 1b. After two weeks the decrease rate was the same for all samples.

~~Fig. 45~~

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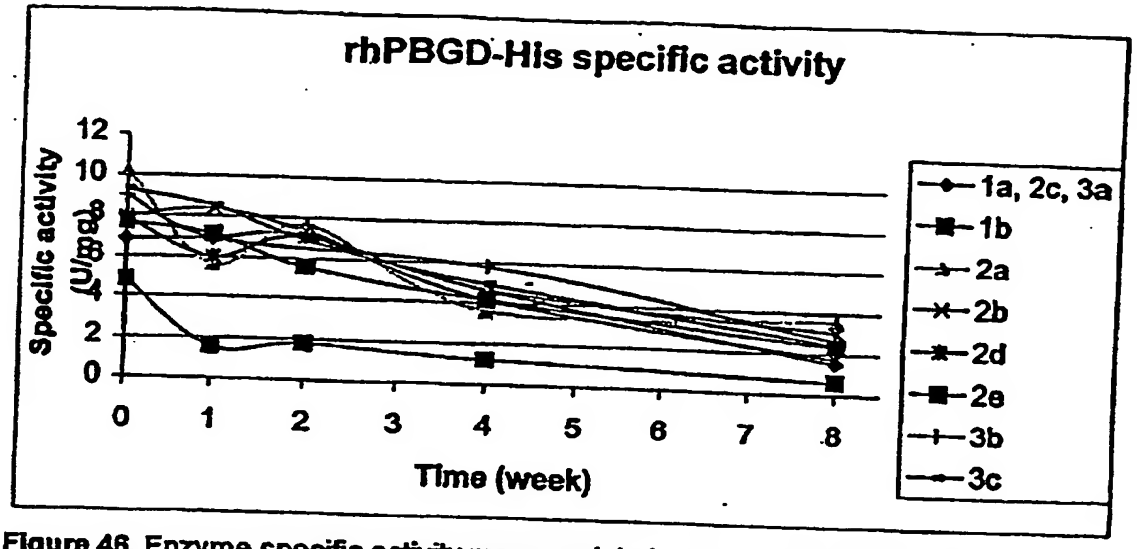

Figure 46. Enzyme specific activity measured during 8 weeks at 40°C. The activity was measured using the enzyme activity assay and the protein concentration was measured using HPLC.

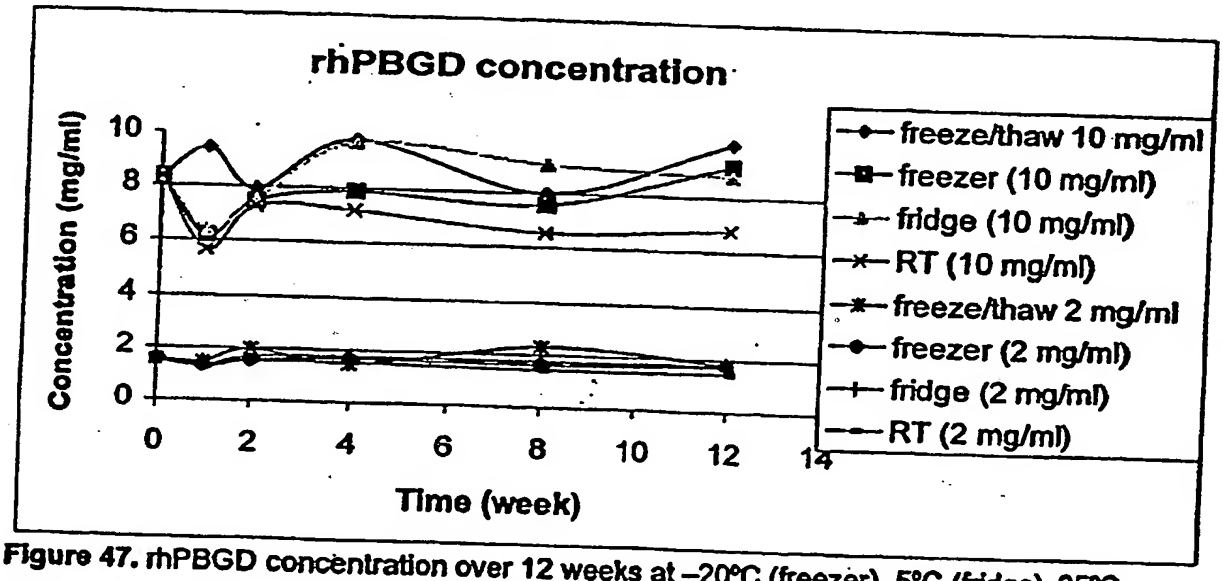

Figure 47. rhPBGD concentration over 12 weeks at –20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling. The measurement was performed using HPLC.

~~Fig. 46~~

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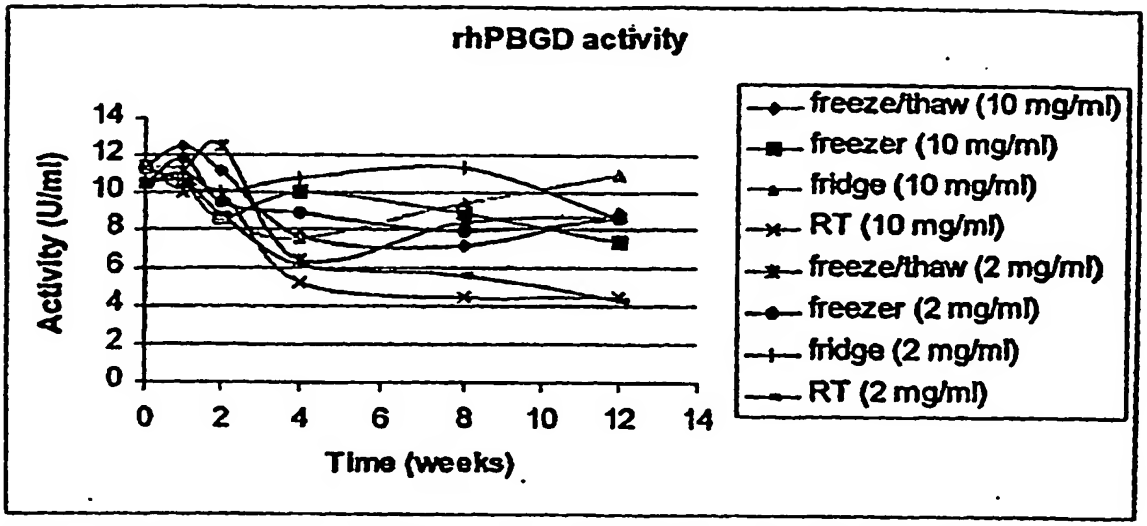

Figure 48. rhPBGD activity over 12 weeks at –20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling.

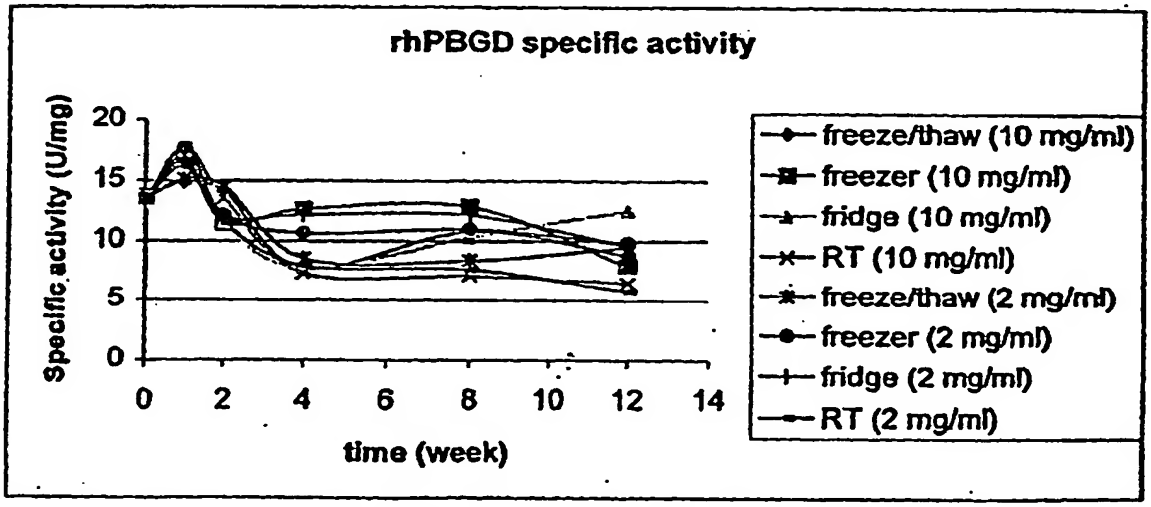

Figure 49. rhPBGD specific activity over 12 weeks at –20°C (freezer), 5°C (fridge), 25°C (RT) and freeze/thawed at each sampling. Measurements were performed using enzyme activity assay and HPLC.

~~Fig. 47~~

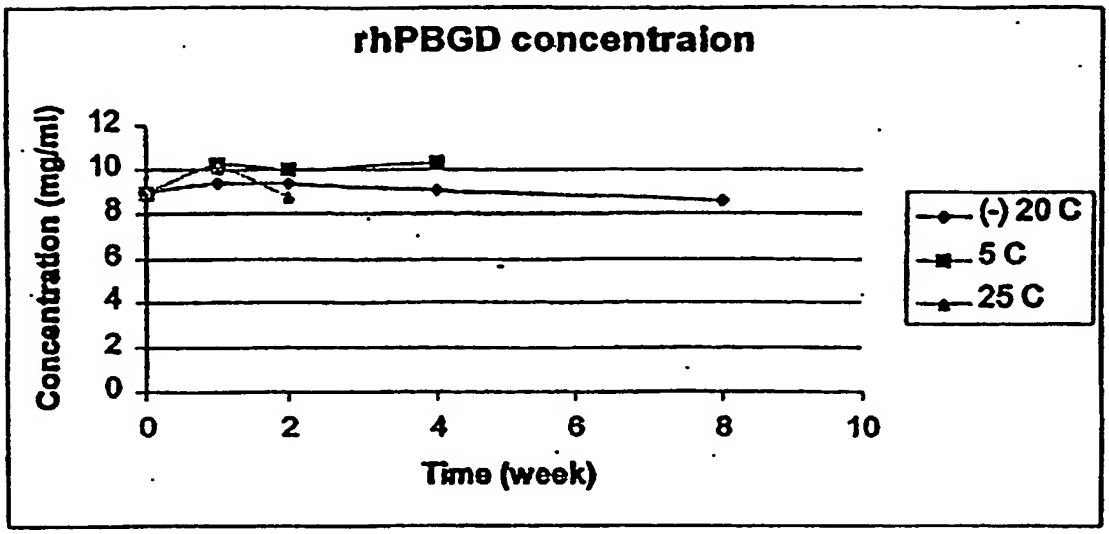

Figure 50. rhPBGD concentration measured over 8 weeks using BCA.

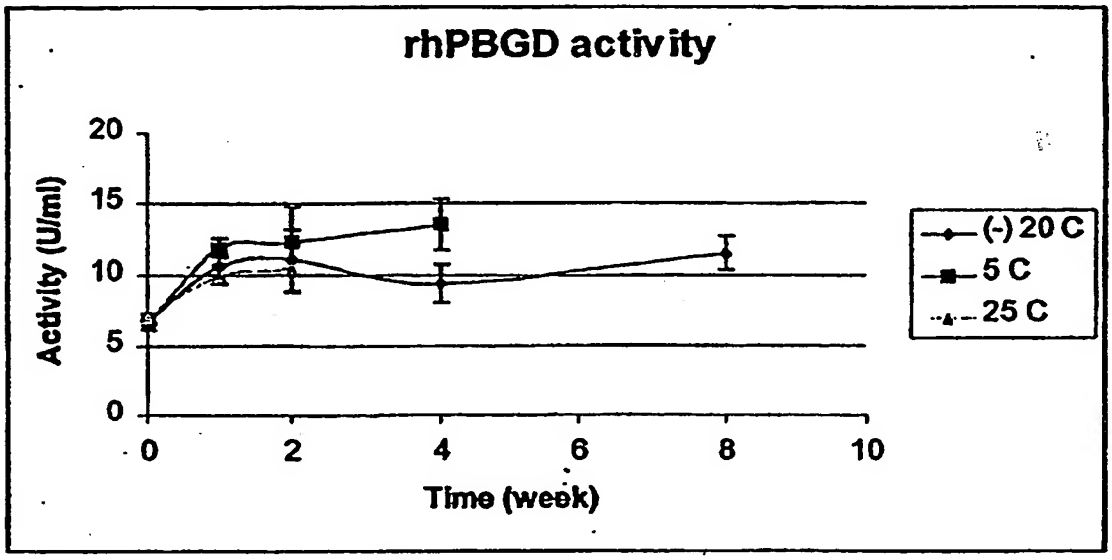

Figure 51. The rhPBGD activity measured over 8 weeks. The stability study has been performed under nitrogen at –20°C ± 5°C, 5°C ± 3°C and at 25°C ± 2°C.

Fig. 48

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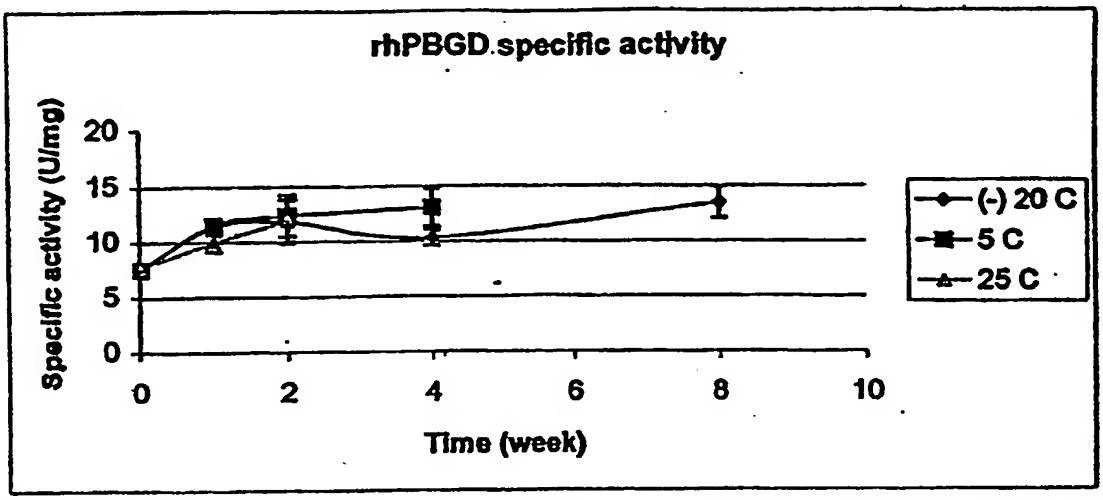

Figure 52. The specific rhPBGD activity measured using the enzyme activity assay and BCA protein concentration assay. The stability study has been performed under nitrogen at –20°C ± 5°C, 5°C ± 3°C and at 25°C ± 2°C.

Fig. 49